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Preparation of 3-Pyridols from Furans

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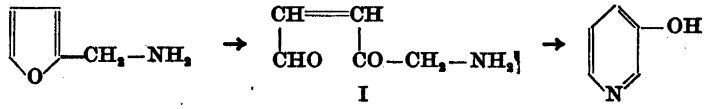
A new method for the preparation of 3-pyridols from 2-(α-aminoalkyl)-furans or their acyl derivatives is described.

Many furans are under certain conditions oxidized to unsaturated 1,4-dicarbonyl compounds or their equivalent [1]. When oxidized in this way, furfurylamine should give a dicarbonylamine having the structure I. Since the double bonds in I are *cis*oid one might expect such a compound to condence intramolecularly to yield 3-pyridol.

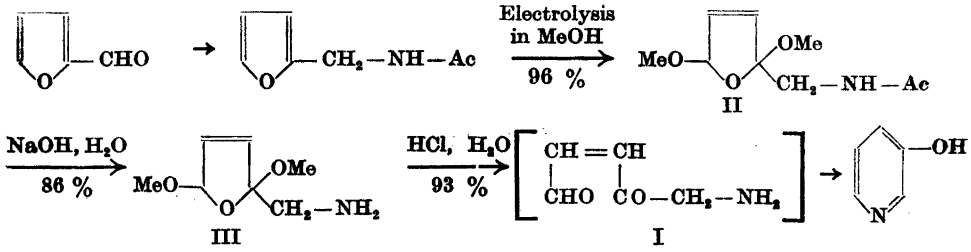


We have indeed found that the transformation of furfurylamine to 3-pyridol is feasible and have worked out reaction conditions, which may be generally used for the preparation of 3-pyridols from 2-(α-aminoalkyl)-furans*. The latter, in turn, are easily obtained by reductive amination of furan-2-carboxaldehydes or furan-2-ketones.

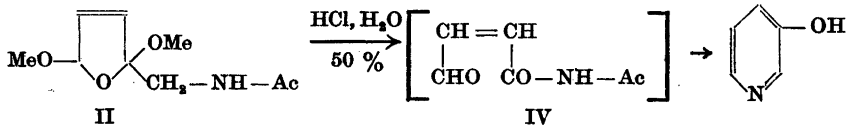
Direct oxidations of furans to unsaturated dicarbonyl compounds are usually difficult to carry out in good yields. If, however, the furans are first electrolytically methoxylated to yield 2,5-dimethoxy-2,5-dihydrofurans and these are subsequently hydrolyzed, yields from 60 to 95 % of dicarbonyl compounds can be obtained.

Electrolytic methoxylation of furfurylamine gave only a low yield of the corresponding dimethoxydihydrofuran (these unsuccessful experiments are not described in the experimental part). Probably the amine group interferes with the methoxylation reaction. But, as has been reported previously [2], 2-(acetamidomethyl)-furan gives an almost quantitative yield of 2,5-dimethoxy-2-(acetamidomethyl)-2,5-dihydrofuran II. Alkaline hydrolysis of II gave the desired 2,5-dimethoxy-2-(aminomethyl)-2,5-dihydrofuran III, which by boiling with *N* hydrochloric acid was transformed into 3-pyridol (yield 93 %).

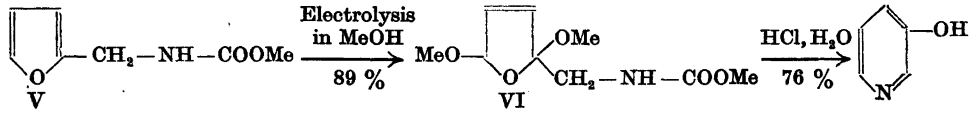
* Patent pending.



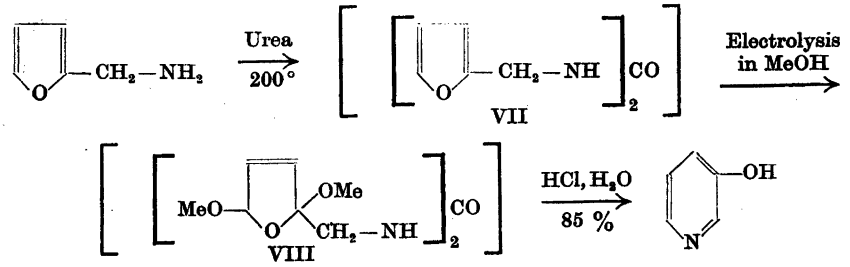
3-Pyridol was also obtained by boiling II with hydrochloric acid, but the yield was only 50 % and the reaction mixture turned dark-red. We believe that the intramolecular condensation of the intermediate dicarbonylamide IV proceeds more slowly than the condensation of the dicarbonylamine I, since the former must hydrolytically lose acetic acid in the course of the condensation. Part of the dicarbonylamide therefore reacts differently due to the prolonged reaction time.



Consistent with this assumption is the fact that an easily hydrolyzed acyl derivative of III, *viz.* the carbamate VI, which was prepared by electrolytic methoxylation of the corresponding furan (V), gave, by boiling with hydrochloric acid, a higher yield of 3-pyridol (76 %).



The above yields of 3-pyridol based upon furfurylamine were 73, 46 and 55 %, respectively. However, by proceeding through *sym*-difurfurylurea VII and the corresponding dimethoxydihydrofuran VIII, an 85 % yield of 3-pyridol was obtained. This reaction sequence, in which the intermediates need not be isolated pure, represents the most convenient way of preparing 3-pyridol from furfurylamine.

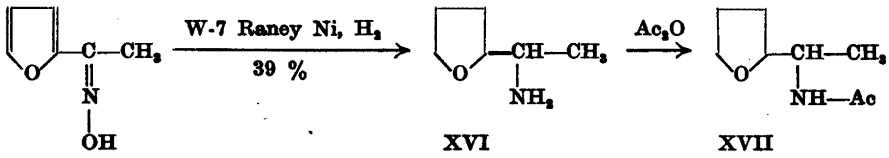


In order to illustrate the general applicability of the new method 2-methyl-3-pyridol was prepared from 2-acetylfuran oxime by the following reactions:

Routes to 2-methyl-3-pyridol from 2-acetylfuran.

The structure of compounds III, V—VI and VIII—XV follows from the methods of their preparation and from analyses. They have all been prepared by known reactions and in only one case, *viz.* the reductive amination of 2-acetylfuran to 2-(α-aminoethyl)-furan (XII) were any difficulties encountered. Under the same conditions as those used for the preparation of furfurylamine from furfural and ammonia, no reaction took place, and attempts to catalytically reduce 2-acetylfuran oxime showed that it was difficult to reduce the oxime group without at the same time reducing the furan nucleus (these experiments are not described in the experimental part). Only when reduced with palladium charcoal in the presence of hydrochloric acid (method of Hartung [3]) did the oxime give a 47 % yield of the amine. Furthermore it was found that reduction of 2-acetylfuran oxime in acetic anhydride with Raney nickel gave a 64 % yield of 2-(α-acetamidoethyl)-furan (IX), in contradiction to the statement of Adkins and Shriner [4] that this catalyst cannot be used in acetic anhydride.

2-(α-Aminoethyl)-tetrahydrofuran (XVI) was prepared by catalytic reduction of 2-acetylfuran oxime with the very active W-7 Raney nickel catalyst. Acetylation of XVI gave the expected 2-(α-acetamidoethyl)-tetrahydrofuran XVII. Refractive indices of these compounds were of value in following the course of the above reductions.



EXPERIMENTAL

Microanalyses by E. Boss, K. Glens and G. Cornali

2,5-Dimethoxy-2-(aminomethyl)-2,5-dihydrofuran (III). 2,5-Dimethoxy-2-(acetamidomethyl)-2,5-dihydrofuran (II, mixture of the *cis* and the *trans* isomer as obtained by the synthesis [2]) (6.96 g, 0.034 mole) was heated under reflux (16 hr) with sodium hydroxide (3 *N*, 100 ml). The mixture was continuously extracted with ether overnight and the amine distilled. The yield was 4.72 g (86 %) of III (colorless liquid, b.p.$_{12}$ 95—96°, n_D^{25} 1.4589).

$C_6H_7ON(OCH_3)_2$ (159.2) Calc. C 52.8 H 8.2 N 8.8 OCH_3 39.0
Found » 53.1 » 8.3 » 9.0 » 39.4

3-Pyridol from III. III (1.94 g, 0.012 mole) was heated under reflux (15 min) with hydrochloric acid (*N*, 22 ml) and the colorless solution evaporated to dryness in a vacuum. The crystalline residue was dissolved in ethanol and the hydrochloride precipitated with ether. The yield was 1.49 g (93 %) of 3-pyridol hydrochloride (almost white crystals, m.p. 105—107° after sintering from 102° (Hershberg apparatus, corr.)).

C_5H_4ONCl (131.6) Calc. C 45.6 H 4.6 N 10.7 Cl 27.0
Found » 45.4 » 4.5 » 10.7 » 26.6

Sublimation (150°/0.2 mm) gave perfectly white crystals, m.p. 106—109° (sintering from 102°). 3-Pyridol hydrochloride has previously been prepared by Fischer and Renouf [5] and by Kao [6], but no physical constants were reported.

3-Pyridol from II. The crystalline isomer of II (4.04 g, 0.020 mole) was heated under reflux (15 min) with hydrochloric acid (*N*, 22 ml) and the dark-red solution evaporated to dryness in a vacuum. To the semisolid black residue containing 3-pyridol hydrochloride was added anhydrous potassium carbonate (2.0 g) and so much water, that the total volume was about 10 ml. The resulting suspension was continuously extracted with ether.

Evaporation of the ether and crystallization of the light-brown residue from benzene gave 0.92 g (48 %) of 3-pyridol (m.p. 125—126°). Sublimation did not change the m.p.

C_5H_5ON (95.1) Calc. C 63.2 H 5.3 N 14.7
Found » 63.3 » 5.3 » 14.4

A similar synthesis starting from the liquid isomer of II gave 0.98 g (52 %) of 3-pyridol (m.p. 125—126°).

Found C 63.0 H 5.3 N 14.5

2-(Carbomethoxyamidomethyl)-furan (V). V was prepared from furfurylamine [7,8] (9.7 g) according to the directions of Hartman and Brethen [9]. The yield was 12.7 g (82 %) of V (colorless liquid, b.p.$_{10}$ 122—123°, n_D^{25} 1.4851).

$C_6H_6O_2N(OCH_3)$ (155.2) Calc. C 54.2 H 5.9 N 9.0 OCH_3 20.0
Found » 54.3 » 6.2 » 8.8 » 19.9

2,5-Dimethoxy-2-(carbomethoxyamidomethyl)-2,5-dihydrofuran (VI). VI was prepared from V (31.0 g, 0.20 mole) by electrolytic methoxylation as described previously for the preparation of II [2] (11.8 amp-hr (110 %) were used). The yield was 38.7 g (89 %) of VI (colorless liquid, b.p.$_{0.3}$ 104—107°, n_D^{25} 1.4652).

$C_6H_6O_2N(OCH_3)_3$ (217.2) Calc. C 49.8 H 7.0 N 6.5 OCH_3 42.9
Found » 49.5 » 6.8 » 6.9 » 42.3

3-Pyridol from VI. VI (4.34 g, 0.020 mole) was heated under reflux with hydrochloric acid (*N*, 22 ml) and the light-brown solution worked up as above. The yield was 1.45 g (76 %) of 3-pyridol (m.p. 125—126°).

Found C 63.1 H 5.3 N 14.4

3-Pyridol from furfurylamine. Furfurylamine (19.4 g, 0.20 mole) and urea (commercial product, 6.60 g, 0.11 mole) were mixed and heated under reflux (1 hr) in an oil bath (190—210°) (method of Davis and Blanchard [10]). Ammonia was evolved. The light-brown crystalline reaction product was dissolved in a solution of ammonium bromide (5.00 g) in methanol (260 ml) and the resulting solution electrolyzed in the usual way [2] (11.9 amp-hr (111 %) were used). After electrolysis, the liquid in the cell was almost colorless in the upper part of the cell and yellow near the bottom. The liquid was poured into a solution of sodium methoxide (from 1.20 g of sodium) in methanol (20 ml) and the methanol and the ammonia evaporated in a vacuum. The semisolid residue was heated under reflux (15 min) with hydrochloric acid (*N*, 240 ml) and the clear yellowish-brown solution evaporated in a vacuum to dryness. Potassium carbonate (22 g) and water (75 ml) were added and the 3-pyridol isolated as above. The yield was 16.1 g (85 %) of 3-pyridol (m.p. 124—127°).

Found C 63.1 H 5.1 N 14.9

In separate experiments *sym*-difurfurylurea (VII) and one of the 3 possible stereoismers of *sym*-(2,5-dimethoxy-2,5 dihydro-2-furfuryl) urea (VIII) were isolated in a pure state.

VII was obtained as white crystals from ethanol (m.p. 129—131°). Further crystallization did not change the m.p. (previously found 128° [11]).

$C_{11}H_{12}O_3N_2$ (220.2) Calc. C 60.0 H 5.5 N 12.7
Found » 60.2 » 5.2 » 12.8

VIII was obtained as white crystals from water (m.p. 149—151°). After recrystallization from water the m.p. was raised to 151—152°.

$C_{11}H_{12}O_3N_2(OCH_3)_4$ (344.4) Calc. C 52.3 H 7.0 N 8.1 OCH_3 36.1
Found » 52.4 » 7.1 » 8.4 » 35.3

2-(α-Acetamidoethyl)-furan (IX). 2-Acetylfuran oxime (12.5 g) and acetic anhydride (30 ml) were shaken (1 hr) with Raney nickel (1.6 g of a 2 months old sample) under hydrogen (100 atm, 70—80°). The reaction product was isolated by distillation. The yield was 9.8 g (64 %) of IX (pale-yellow liquid, b.p.$_{0.1}$ 86—89°, n_D^{25} 1.4922).

$C_6H_3ON(COCH_3)$ (153.2) Calc. C 62.7 H 7.2 N 9.1 $COCH_3$ 28.1
Found » 62.7 » 7.5 » 9.4 » 28.5

IX was also prepared by acetylation of 2-(α-aminoethyl)-furan (XII) with acetic anhydride (b.p.$_{12}$ 143—144°, n_D^{25} 1.4929).

IX crystallized after standing for some time. Crystallization from ether — petroleum ether gave white crystals with the m.p. 52—53°. Another crystallization did not change the m.p.

Found C 62.4 H 7.2 N 9.3 $COCH_3$ 27.6

2,5-Dimethoxy-2-(α-acetamidoethyl)-2,5-dihydrofuran (X). IX (2.00 g, 0.013 mole) and ammonium bromide (0.30 g, 0.0031 mole) were dissolved in methanol (analytical grade, 20 ml) and the solution electrolyzed in the small cell described previously [12] (temperature of cooling-bath −20°).

Time hr	Current amp	Potential across the cell during electrolysis (volt)	Ampere hours (per cent of theoretical amount)	
0.1	0.8	6.2	0.07	(10 %)
0.3	0.7	6.2	0.25	(36 %)
0.5	0.7	6.2	0.34	(49 %)
1.0	0.6	6.3	0.66	(95 %)
1.2	0.5	6.4	0.77	(110 %)

After electrolysis, the pale-yellow liquid was poured into a solution of sodium methoxide (from 73 mg (0.0032 mole) of sodium) in methanol (3 ml) and the methanol and the ammonia evaporated in a vacuum. Anhydrous ether (50 ml) was added, a precipitate of sodium bromide removed by filtration and the filtrate distilled in a vacuum. The yield was 2.47 g (88 %) of X (colorless, viscous oil, $b.p._{0.1}$ 110−115°, n_D^{25} 1.4739).

$C_6H_8ON(OCH_3)_2(COCH_3)$ (215.2) Calc. C 55.8 H 8.0 N 6.5 OCH_3 28.8 $COCH_3$ 20.0
Found » 56.1 » 7.9 » 6.6 » 28.9 » 20.3

2,5-Dimethoxy-2-(α-aminoethyl)-2,5-dihydrofuran (XI). XI was prepared from X (2.15 g) as described for the preparation of III (refluxing time 38 hr). The yield was 1.39 g (80 %) of XI (colorless liquid, $b.p._{12}$ 90−92°, n_D^{25} 1.4565).

$C_6H_9ON(OCH_3)_2$ (173.2) Calc. C 55.5 H 8.7 N 8.1 OCH_3 35.8
Found » 55.5 » 9.0 » 8.3 » 35.8

2-Methyl-3-pyridol from XI. This preparation was carried out with XI (1.10 g, 0.0064 mole) as described for the preparation of 3-pyridol from III. The yield was 0.88 g (95 %) of 2-methyl-3-pyridol hydrochloride (white crystals, m.p. 223−224°).

C_6H_8ONCl (145.6) Calc. C 49.5 H 5.5 N 9.6 Cl 24.4
Found » 49.6 » 5.7 » 9.9 » 24.8

The free base was prepared from the hydrochloride in the usual way and obtained as yellowish-white crystals (m.p. 169−170°, previously found 167−168° [13] and 163−165° [14]).

C_6H_7ON (109.1) Calc. C 66.0 H 6.5 N 12.8
Found » 66.2 » 6.6 » 13.0

Sublimation (150°/0.05 mm) gave white crystals (m.p. 169−170°).

2-(α-Aminoethyl)-furan (XII) (directions of Hartung [3]). 2-Acetylfuran oxime (15.6 g, 0.12 mole) was dissolved in absolute ethanol (200 ml), to which had been added acetyl chloride (26.5 ml, 0.37 mole), and the solution was shaken (4 hr) with palladium charcoal (10 %, 6.5 g) under hydrogen (15 atm). After filtration the solvent was evaporated in a vacuum, the residue made alkaline with sodium hydroxide (50 %) and continuously extracted with ether. Distillation through a short packed column gave 6.5 g (47 %) of XII (colorless liquid, $b.p._{760}$ 148−149°, n_D^{25} 1.4748).

C_6H_9ON (111.1) Calc. C 64.8 H 8.2 N 12.6
Found » 64.6 » 8.3 » 12.5

Picrate. The m.p. was 184−186° (dec.) after two crystallizations from ethanol.

$C_{12}H_{12}O_8N_4$ (340.3) Calc. C 42.4 H 3.6 N 16.5
Found » 42.4 » 3.7 » 16.8

Phenylthiourea derivative. The m.p. was 101−102° after two crystallizations from ethanol.

$C_{13}H_{14}ON_2S$ (246.3) Calc. C 63.4 H 5.7 N 11.4
Found » 64.0 » 5.7 » 11.2

2-(α-Carbomethoxyamidoethyl)-furan (XIII). 2-Acetylfuran oxime (12.5 g, 0.10 mole) was hydrogenated as described above for the preparation of XII. The reaction mixture was filtered and evaporated to dryness in a vacuum, finally at 0.1 mm (50−55°). The reddish-brown residue was dissolved in water (20 ml), extracted with ether and carbo-

methoxylated with methyl chloroformate (0.10 mole) according to the directions of Hartman and Brethen [9]. The yield was 9.2 g (54 %) of XIII (colorless liquid, b.p.$_{0.1}$ 72—75°, n_D^{25} 1.4805).

$C_7H_8O_2N(OCH_3)$ (169.2)	Calc.	C 56.8	H 6.6	N 8.3	OCH_3	18.3
	Found	» 57.1	» 7.0	» 8.6	»	18.2

Since the yield, from the reduction of 2-acetylfuran oxime, of actually isolated 2-(α-aminoethyl)-furan was only 47 %, the carbomethoxylation must have been almost quantitative and the carbamate furthermore easier to isolate than the amine.

2,5-Dimethoxy-2-(α-carbomethoxyamidoethyl)-2,5-dihydrofuran (XIV). XIV was prepared from XIII (11.6 g, 0.068 mole) by electrolytic methoxylation as described previously for the preparation of II[2] (4.1 amp-hr (110 %) were used). The yield was 13.8 g (88 %) of XIV (colorless oil, b.p.$_{0.2}$ 98—102°, n_D^{25} 1.4649).

$C_7H_8O_2N(OCH_3)_3$ (231.2)	Calc.	C 51.9	H 7.4	N 6.1	OCH_3	40.3
	Found	» 52.2	» 7.7	» 5.9	»	40.5

2-Methyl-3-pyridol from XIV. XIV (2.31 g, 0.010 mole) and hydrochloric acid (*N*, 100 ml) were heated under reflux (1.5 hr). After decantation from a small amount of an insoluble oil the mixture was evaporated in a vacuum to dryness. The residue was dissolved in methanol (about 5 ml) and the hydrochloride precipitated with ether (100 ml). The yield was 1.23 g (85 %) of crude 2-methyl-3-pyridol hydrochloride (light-brown powder). 200 mg of this product was purified by sublimation (195°/0.05 mm). The yield was 170 mg (72 %) of almost white crystals (m.p. 223—225°).

Found	C 49.6	H 5.5	N 9.3	Cl 24.7

2-(α-Ureidoethyl)-furan (XV[10]*).* XII (6.12 g, 0.055 mole) was dissolved in hydrochloric acid (5.8 *N*, 9.5 ml, 0.055 mole) under cooling, a solution of urea (3.45 g, 0.057 mole) in water (16 ml) was added and the mixture heated under reflux (16 hr). After cooling, the crystalline precipitate was removed by filtration, washed twice with water and dried. The yield was 4.24 g (50 %) of crude XV (light-brown powder, m.p. 91—103°). 200 mg was crystallized from ethyl acetate-ether, yielding 140 mg (35 %) of XV (white crystals, m.p. 114—116°). Sublimation did not change the m.p.

$C_7H_{10}O_2N_2$ (154.2)	Calc.	C 54.5	H 6.5	N 18.2
	Found	» 54.5	» 6.5	» 18.2

2-Methyl-3-pyridol from XV. XV (340 mg, 0.0022 mole) and ammonium bromide (150 mg, 0.0015 mole) were dissolved in methanol (analytical grade, 10 ml) and the mixture electrolyzed in the small cell described previously [12] (6.4—6.6 v, 0.4 amp, 0.35 hr). 0.13 amp-hr (0.0024 faraday) was passed through the cell. After electrolysis the pale-yellow liquid was poured into a solution of sodium methoxide (from 37 mg of sodium (0.0016 mole)) in methanol (3 ml) and the solvent evaporated in a vacuum. The residue was heated under reflux (15 min) with hydrochloric acid (*N*, 22 ml) and the pyridol isolated as described for the preparation of 3-pyridol from II. The crude methylpyridol was converted to the hydrochloride by addition of concentrated hydrochloric acid and evaporation in a vacuum to dryness. The hydrochloride was then purified by precipitation from ethanol — ether (yield 210 mg) followed by sublimation (190°/0.05 mm). The yield was 200 mg (62 %) of 2-methyl-3-pyridol hydrochloride (m.p. 225—227°).

Found	C 49.4	H 5.2	N 9.6	Cl 24.7

2-(α-Aminoethyl)-tetrahydrofuran (XVI). 2-Acetylfuran oxime (6.0 g) was dissolved in methanol (50 ml) and the solution was shaken (16 hr) with W-7 Raney nickel catalyst [15] (1 g) under hydrogen (100—50 atm). After filtration the solvent was distilled through a short packed column and the residue distilled further in a vacuum without the column. The yield was 2.17 g (39 %) of XVI (colorless liquid, b.p.$_{13}$ 47—48°, n_D^{25} 1.4489).

$C_6H_{13}ON$ (115.2)	Calc.	C 62.6	H 11.4	N 12.2
	Found	» 62.6	» 11.5	» 12.0

Phenylthiourea derivative. The m.p. was 111—115° after two crystallizations from ethanol.

$C_{13}H_{18}ON_2S$ (250.4)	Calc.	C 62.4	H 7.3	N 11.2
	Found	» 62.5	» 7.3	» 11.4

Acetyl derivative (XVII). XVII was obtained as a colorless liquid (b.p.$_{0.4}$ 97—101°, n_D^{25} 1.4722).

$C_6H_{12}ON(COCH_3)$ (157.2)	Calc.	C 61.1	H 9.6	N 8.9	$COCH_3$	27.4
	Found	» 61.5	» 9.7	» 8.6	»	26.8

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* *Editor's note:* On the request of the authors the publication of this and the following six papers by the same group has been postponed.

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Electrolytic Methoxylation of 2-Acetylfuran

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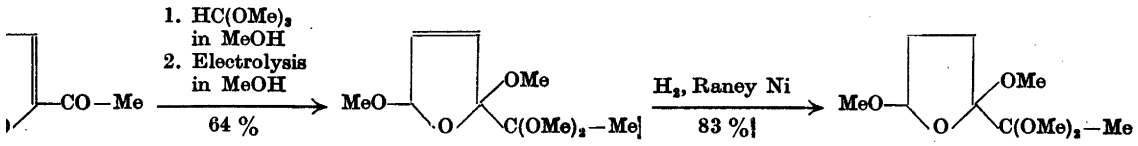
2-Acetylfuran has been methoxylated by transformation into the dimethyl ketal and electrolysis in methanol. Reaction of the resulting 2,5-dimethoxy-2-(α,α-dimethoxyethyl)-2,5-dihydrofuran (I) with an aqueous solution of hydroxylamine hydrochloride gave 2-methyl-3,6-pyridinediol-1-oxide (IV). This compound has previously been prepared by Vargha, Ramonczai and Bite, who believed it to be an aldoxime of 2-hexene-4,5-dione-1-al(III). I was catalytically hydrogenated to 2,5-dimethoxy-2-(α,α-dimethoxyethyl)-tetrahydrofuran (II), which by heating with dilute hydrochloric acid yielded pyrocatechol.

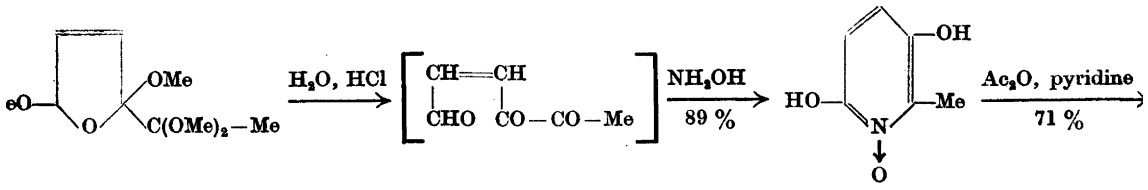
As part of an investigation of the electrolytic methoxylation of furans, reaction conditions have been worked out for the methoxylation of 2-acetylfuran. The furan is first heated under reflux with methanol, methyl orthoformate and a trace of *p*-toluenesulfonic acid, and the resulting solution, which probably contains 2-acetylfuran dimethyl ketal, is then electrolyzed. Analyses showed the reaction product to be the expected 2,5-dimethoxy-2-(α,α-dimethoxyethyl-2,5-dihydrofuran (I). Catalytic hydrogenation of I gave the corresponding tetrahydrofuran II.



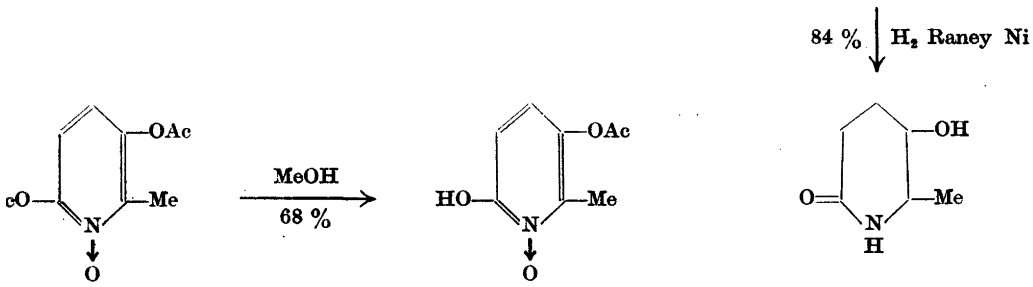
I II

By reaction of I with an aqueous solution of hydroxylamine hydrochloride an 89 % yield of a compound $C_6H_7O_3N$ (m.p. 236°), which was soluble in alkali, sparingly soluble in water and alcohol and insoluble in ether, was obtained. It gave a strong violet ferric chloride reaction. Acetylation of this compound yielded a diacetate, which was transformed by solution in methanol into a monoacetate. The diacetate gave a red ferric chloride reaction after some seconds, while the monoacetate gave the same reaction immediately. Catalytic

hydrogenation of the $C_6H_7O_3N$-compound yielded a compound $C_6H_{11}O_2N$, which gave no ferric chloride reaction. Assuming the $C_6H_7O_3N$-compound to be 2-methyl-3,6-pyridinediol-1-oxide (IV), these experiments may be explained as shown below.

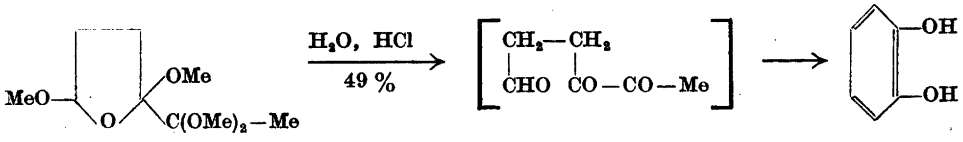


I III IV



V VI VII

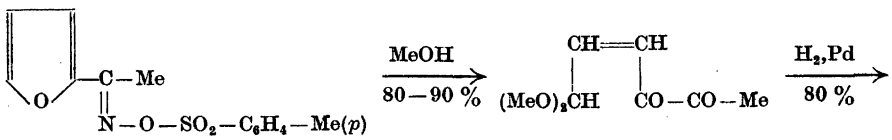
By heating of II with dilute hydrochloric acid, pyrocatechol is formed in a 49 % yield. Evidently hydrolysis to hexane-4,5-dione-1-al (VIII) followed by intramolecular condensation takes place.



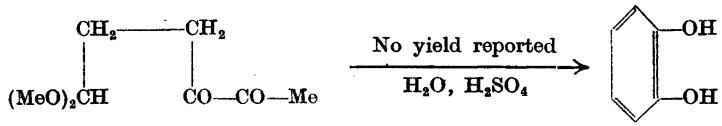
II VIII

Vargha, Ramonczai and Bite[1] have reported that treatment of the *p*-toluenesulfonate of 2-acetylfuran oxime (IX) with methanol results in the formation of a liquid $C_8H_{12}O_4$, which may be catalytically hydrogenated to another liquid $C_8H_{14}O_4$. The $C_8H_{12}O_4$-liquid gave, with an aqueous solution of hydroxylamine hydrochloride, a compound $C_6H_7O_3N$ with properties similar to our $C_6H_7O_3N$-compound (2-methyl-3,6-pyridinediol-1-oxide (IV)). The $C_8H_{14}O_4$-liquid gave pyrocatechol by heating with dilute sulfuric acid. These reactions were explained by Vargha, Ramonczai and Bite as shown below.

The $C_6H_7O_3N$-compound was assumed to be an aldoxime of 2-hexene-4,5-dione-1-al (III).



IX X



XI

Vargha, Ramonczai and Bite did not report any spectra, refractive indices or methoxy values for their liquids, so that it is not certain whether these actually have been pure compounds with the proposed structures (X and XI) or not. It is, however, apparent from the published data, and also in conformity with our findings, that the liquids as claimed are derivatives of 2-hexene-4,5-dione-1-al (III) and hexane-4,5-dione-1-al (VIII), respectively. The $C_6H_7O_3N$-compound, which evidently is identical with our $C_6H_7O_3N$-compound, must on the other hand be 2-methyl-3,6-pyridinediol-1-oxide (IV).

EXPERIMENTAL

Microanalyses by E. Boss and K. Glens

2,5-Dimethoxy-2-(α,α-dimethoxyethyl)-2,5-dihydrofuran (I). 2-Acetylfuran (3.30 g, 0.030 mole) and methyl orthoformate (3.50 g, 0.033 mole) was added to a solution of *p*-toluenesulfonic acid (8.6 mg) in anhydrous methanol (10 ml) and the mixture heated under reflux (10 min). After cooling a solution of ammonium bromide (0.60 g) in methanol (30 ml) was added and the mixture electrolyzed in the small cell described previously [2] (temperature of cooling-bath −18°).

Time hr	Current amp	Potential across the cell during electrolysis volt	Ampere hours (per cent of theoretical amount)	
0.1	1.0	4.8	0.09	(6 %)
0.5	0.9	5.0	0.43	(27 %)
1.0	0.8	5.1	0.81	(50 %)
2.5	0.6	5.2	1.68	(104 %)

After electrolysis the yellow liquid was poured into a solution of sodium methoxide (from sodium (147 mg) in methanol (5 ml)) and the methanol and the ammonia evaporated in a vacuum. Anhydrous ether (50 ml) was added, the precipitate removed by filtration and the filtrate distilled in a vacuum.

Fraction (g)	B.p.$_{14}$ °C	n_D^{25}
1 (0.30)	108–112	1.4502
2 (3.37)	112–113	1.4498
3 (0.48)	113–115	1.4499

The yield (all fractions) was 4.15 g (64 %, current efficiency 62 %) of I (colorless liquid). A portion of fraction 2 was analyzed.

$C_6H_8O(OCH_3)_4$ (218.2)	Calc.	C	55.0	H	8.3	OCH_3	56.9
	Found	»	55.1	»	8.2	»	55.4

All fractions gave a positive Beilstein test for halogens, thus indicating, that the product is contaminated by a small amount of some bromine-containing impurity.

2,5-Dimethoxy-2-(α,α-dimethoxyethyl)-tetrahydrofuran (II). I (3.00 g), anhydrous methanol (15 ml) and potassium hydroxide (11 mg) were shaken (4 hr) with Raney nickel (0.3 g) under hydrogen (100 atm). The product was isolated by distillation.

Fraction (g)	B.p.$_{14-15}$ °C	n_D^{25}	Calc. for $C_6H_8O(OCH_3)_4$ (220.3)		
			C 54.5%	H 9.2%	OCH_3 56.4%
1 (0.31)	116	1.4390	54.2	9.2	54.0
2 (2.21)	118–119	1.4387	54.7	9.0	55.3

The yield (both fractions) was 2.52 g (83 %) of II (colorless liquid).

2-Methyl-3,6-pyridinediol-1-oxide (IV). I (0.44 g, 0.002 mole) was dissolved in water (5 ml) and the solution added to a solution of hydroxylamine hydrochloride (0.21 g, 0.003 mole) in water (2 ml). The mixture was left standing for 15 minutes whereby white crystals precipitated. The crystals were removed by filtration, washed twice with water and dried. The yield was 0.25 g (89 %) of IV (white needles, m. p. 220–230° (dec.) (Hershberg apparatus, corr.)) Crystallization from ethanol gave 0.21 g (75 %), m. p. in evacuated tube 234–236° (dec.). Vargha, Ramonczai and Bite[1] found m. p. 236° for their $C_6H_7O_2N$-compound.

$C_6H_7O_2N$ (141.1)	Calc.	C	51.1	H	5.0	N	9.9
	Found	»	50.8	»	5.4	»	10.0

IV was sparingly soluble in water and insoluble in ether. It gave a strong violet color with ferric chloride.

2-Methyl-3,6-diacetoxypyridine-1-oxide (V). A mixture of IV (0.42 g), pyridine (5 ml) and acetic anhydride (5 ml) was heated to boiling, left standing for 5 minutes, cooled and evaporated in a vacuum. Addition of ether to the residue gave 0.32 g of V (almost colorless crystals, m. p. 96–98°).

$C_6H_5O_2N(COCH_3)_2$ (225.2)	Calc.	C	53.3	H	4.9	N	6.2	$COCH_3$ 38.2
	Found	»	53.2	»	4.9	»	6.0	» 39.0

From the filtrate another 0.16 g of V (white crystals, m. p. 96—98°) was obtained, the total yield of V thus being 0.48 g (71 %).

Found C 53.4 H 5.2 N 6.2 $COCH_3$ 38.4

V gave a red color with aqueous ferric chloride after some seconds.

2-Methyl-3-acetoxy-6-pyridinol-1-oxide (VI). V (90 mg) and methanol (2 ml) were heated to boiling and twice partly evaporated with ether, whereby white crystals were obtained. The yield was 50 mg (68 %) of VI (white crystals, m. p. 129—131°).

$C_6H_6O_2N(COCH_3)$	(183.2)	Calc.	C	52.5	H	5.0	N 7.7	$COCH_3$ 23.5
		Found	»	52.5	»	5.2	» 7.5	» 23.3

VI gave instantaneously a red color with aqueous ferric chloride.

5-Hydroxy-6-methyl-2-piperidone (VII). IV (1.00 g) and anhydrous methanol (30 ml) were shaken (1 hr) with Raney nickel (0.4 g) under hydrogen (100 atm, 100° C). The Raney nickel was removed by filtration and the filtrate evaporated in a vacuum. The white crystalline residue was crystallized from methanol. The yield was 0.67 g of VII (white crystals, m. p. 178—180°; recrystallization from methanol-ether did not change the m. p.).

$C_6H_{11}O_2N$	(129.2)	Calc.	C	55.8	H	8.6	N 10.9
		Found	»	56.0	»	8.9	» 10.7

Addition of ether to the mother liquor and crystallization from methanol of the crystals obtained gave another 0.10 g of VII (white crystals, m. p. 177—179°), the total yield of VII thus being 0.77 g (84 %).

VII gave no coloration with aqueous ferric chloride.

Acetate of VII. A mixture of VII (100 mg), pyridine (2 ml) and acetic anhydride (2 ml) was heated to boiling and then evaporated in a vacuum. The white crystalline residue was boiled with ether. The yield was 90 mg (68 %) of 5-acetoxy-6-methyl-2-piperidone (white crystals, m. p. 169—172°).

$C_6H_{10}O_2N(COCH_3)$	(171.2)	Calc.	C	56.1	H	7.7	N 8.2	$COCH_3$ 25.1
		Found	»	56.5	»	8.0	» 8.0	» 25.2

Hydrochloride of VII. VII (100 mg) dissolved in methanol (2 ml) and methanol (1 ml), to which had been added acetyl chloride (0.08 ml), were mixed and the hydrochloride precipitated with ether, removed by filtration, washed with ether and dried. The yield was 120 mg (94 %) of 5-hydroxy-6-methyl-2-piperidone hydrochloride (white crystals, m. p. 146—149°).

$C_6H_{12}O_2NCl$	(165.6)	Calc.	C	43.5	H	7.3	N 8.5	Cl 21.4
		Found	»	43.1	»	7.4	» 8.0	» 21.3

Pyrocatechol from II. II (4.56 g) and *N* hydrochloric acid (45 ml) were mixed and heated under reflux (2 hr). The light-brown mixture was cooled and saturated with sodium hydrogen carbonate. A small amount of an insoluble oil was removed and the mixture extracted continuously with ether. The etheral extract was dried with magnesium sulfate and distilled in a vacuum. The yield was 1.12 g (49 %) (light-brown crystals, b. $p._1$ 88—90°, m. p. 97—102°). Crystallization of 200 mg from ether-petroleum ether gave 160 mg of almost white crystals, m. p. 100—103°.

$C_6H_6O_2$	(110.1)	Calc.	C	65.4	H	5.5
		Found	»	65.1	»	5.6

The crystals gave no m. p. depression when mixed with an authentic specimen of pyrocatechol.

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2. Limborg, F. and Clauson-Kaas, N. *Acta Chem. Scand.* **7** (1953) 234.

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New Methods for the Transformation of Furans into Pyridines

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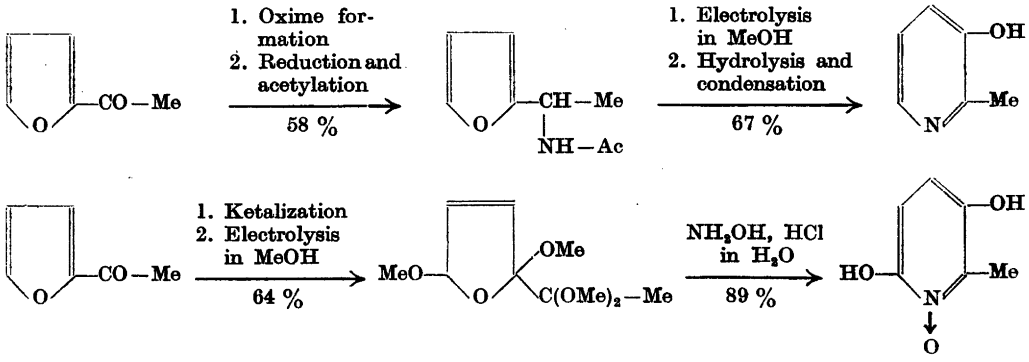
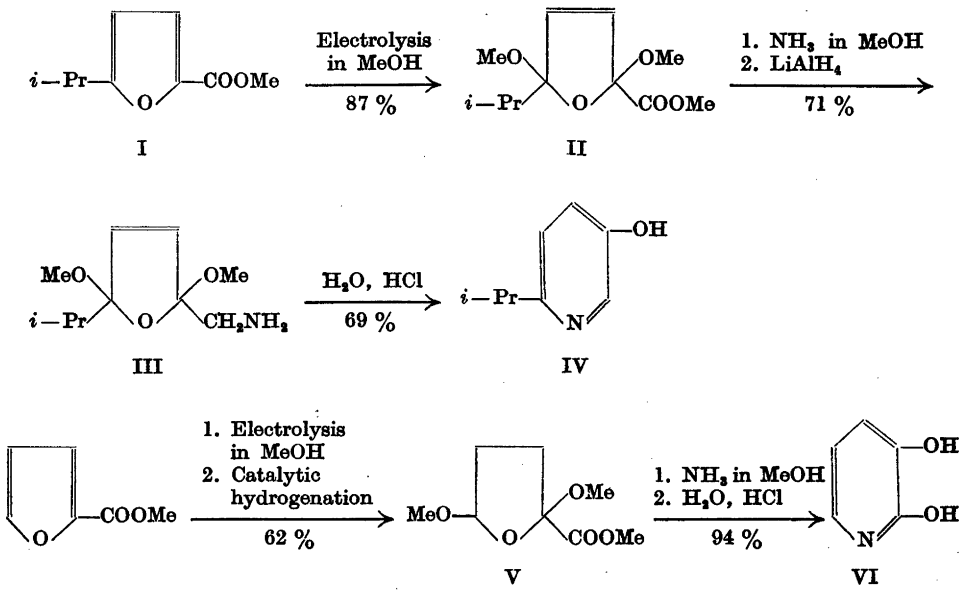
The preparation of 6-*iso*propyl-3-pyridinol (IV) and of 2,3-pyridinediol (VI) from 2-carbomethoxy-5-*iso*propylfuran (I) and methyl furoate, respectively, is described.

Two routes from furan-2-carboxaldehydes or furan-2-ketones to pyridines have recently been reported [1,2]. These may be exemplified by the transformation of 2-acetylfuran into 2-methyl-3-pyridinol and 2-methyl-3,6-pyridinediol-1-oxide, respectively.



Two routes from furan-2-carboxylic acid esters, many of which are easily accessible, to 3-pyridinols and 2,3-pyridinediols, respectively, are described here. The first route is exemplified by the transformation of 2-carbomethoxy-5-*iso*propylfuran (I) into 6-*iso*propyl-3-pyridinol (IV), the second by the transformation of methyl furoate into 2,3-pyridinediol (VI).

The preparation of pyrocatechol from 2-acetylfuran reported in the foregoing article [2] represents a sequence of reactions similar to the reactions described here. The main feature of these transformations of furans into other



compounds with aromatic character is an intramolecular condensation of a straight chain of atoms including a 1,4-dicarbonyl C_4-unit, the latter coming into existence by oxidation of a furan compound. We believe that many other heterocyclic or homocyclic aromatic compounds can be prepared by the same type of reactions by employing suitable 2-substituted furans.

EXPERIMENTAL

Microanalyses by E. Boss and K. Glens

2,5-Dimethoxy-2-aminomethyl-5-isopropyl-2,5-dihydrofuran (III). II[3] (3.45 g, 0.015 mole) was mixed at −50° with a solution of ammonia (2.2 ml, measured at −80°) and water (0.3 ml) in methanol (6 ml) and the resulting solution left standing (7 days) at room temperature and then evaporated in a vacuum from a water-bath (60°). The residue was dissolved in anhydrous ether and evaporated once more to dryness, at last under 0.1 mm, in order to remove traces of water and methanol. The residue was dissolved in ether (90 ml) and the etheral solution added with stirring to a suspension of lithium aluminum hydride (0.97 g, 0.026 mole) in ether (60 ml). After stirring at room temperature for 20 hr the mixture was cooled to −20°, and water (20 ml) and then sodium hydroxide solution (20 %, 25 ml) were added dropwise. The cloudy reaction mixture was continuously extracted with ether, the etheral solution dried with magnesium sulfate and distilled. The yield was 2.13 g (71 %) of III (colorless liquid, b. $p_{\cdot 13-12}$ 121−126°, n_D^{25} 1.4625).

$C_8H_{13}ON(OCH_3)_2$ (201.3)	Calc.	C 59.7	H 9.5	N 7.0	OCH_3 30.8
	Found	» 60.0	» 9.5	» 7.0	» 30.5

6-Isopropyl-3-pyridinol (IV). III (1.50 g, 0.0075 mole) and hydrochloric acid (*N*, 17 ml) were heated under reflux (15 min). The solution was evaporated to dryness in a vacuum and the residual dark-brown oil dissolved in water (10 ml). Potassium carbonate (1.0 g) was added and the solution continuously extracted with ether. A crust of yel-

lowish-white crystals was removed by decantation, washed with ether and dried. The yield was 0.65 g of IV (slightly yellow crystals, m. p. 155—157° (Hershberg apparatus, corr.)).

$C_8H_{11}ON$ (137.2)	Calc.	C	70.0	H	8.1	N	10.2
	Found	»	70.1	»	7.9	»	10.5

The product gave a wine-red ferric chloride reaction. Sublimation (0.06 mm, 135—140°) gave a perfectly white product in an almost quantitative yield (m. p. 156°).

From the etheral solution another 0.06 g of IV was isolated by sublimation (white crystals, m. p. 154—156°), the total yield of IV thus being 0.71 g (69 %).

2,3-Pyridinediol (VI). V[4] (4.75 g, 0.025 mole) was mixed at −50° with a solution of ammonia (3.5 ml, measured at −80°) and water (0.5 ml) in methanol (10 ml) and the resulting solution left standing (4 days) at room temperature and then evaporated in a vacuum from a water-bath. The almost colorless viscous residue was dissolved in a mixture of water (10 ml) and methanol (3 ml). Sulfuric acid (0.1 *N*, 10.0 ml) was added and the solution heated under reflux (20 min). To the hot solution barium hydroxide (0.1 *N*, 10.0 ml) was added, the light yellow suspension evaporated to dryness in a vacuum and the solid residue sublimed (0.07 mm, 145—155°). The yield was 2.60 g (94 %) of VI (m. p. in an evacuated tube 250—52 (dec.)). The product gave the expected strong blue ferric chloride reaction.

$C_5H_5O_2N$ (111.1)	Calc.	C	54.1	H	4.5	N	12.6
	Found	»	54.3	»	4.4	»	12.8

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New Examples of Electrolytic Methoxylation of Furans

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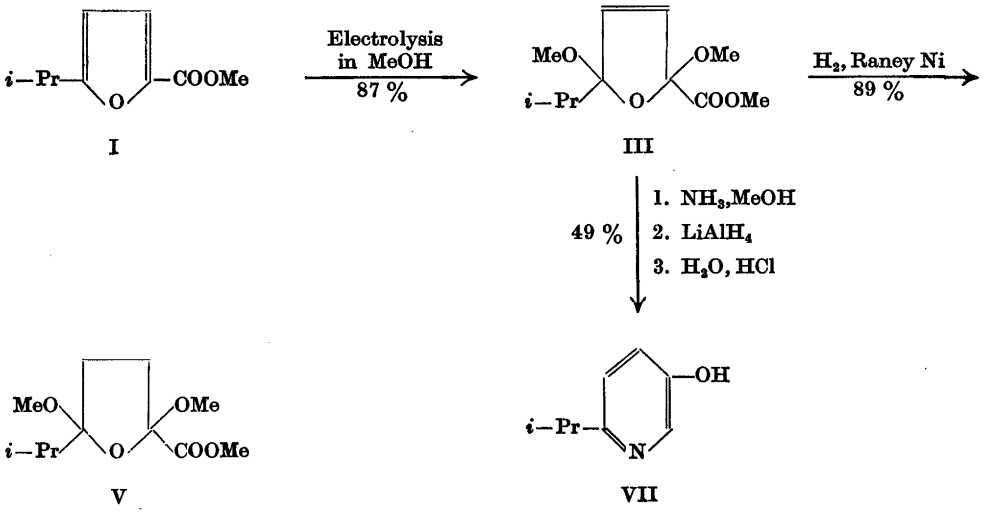
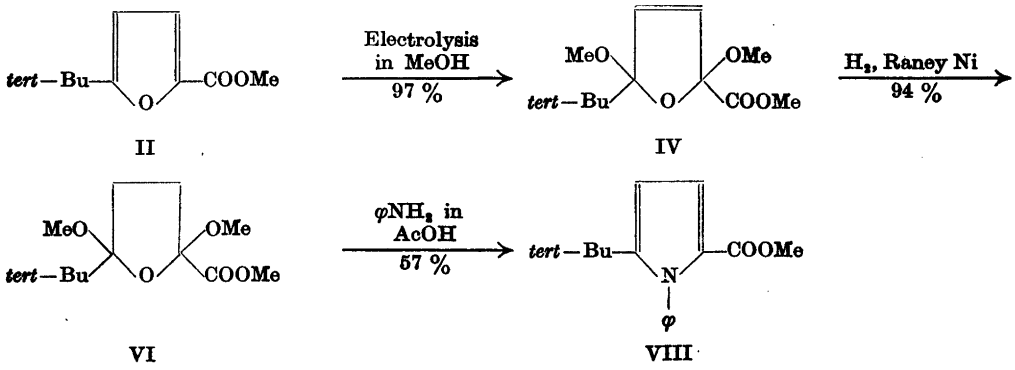
Four new 2,5-dimethoxy-2,5-dihydrofurans have been prepared by electrolytic methoxylation of the corresponding furans.

In connection with an investigation of certain furan compounds[1] four new dimethoxydihydrofurans have been prepared by the electrolytic methoxylation method[2,3]. In all 20 different furans have now been electrolytically methoxylated.

2-Carbomethoxy-5-*iso*propylfuran[4] (I) and 2-carbomethoxy-5-(*tert*butyl)-furan[4] (II) were electrolyzed in a sulfuric acid electrolyte[3]. Both dimethoxydihydrofurans (III and IV) were catalytically hydrogenated to the corresponding tetrahydrofurans (V and VI). The transformation of III into the 3-pyridinol VII[5] proves the structures of III and V. The structures of IV and VI were proved by transformation of VI into the pyrrole VIII (*cf.*[6]).

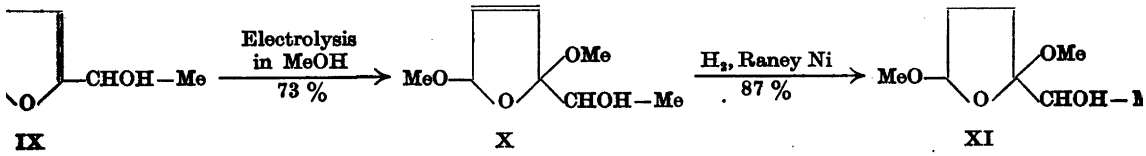


I III V VII

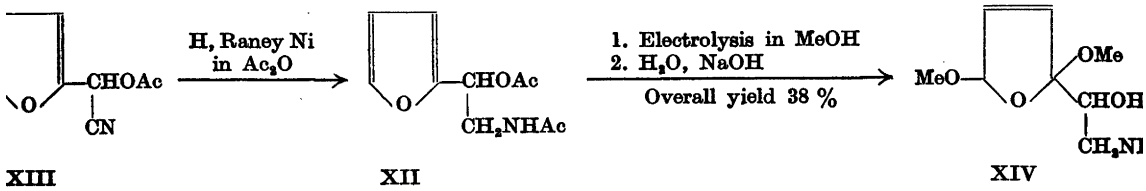


The Zeisel methoxy determination of 1-phenyl-2-carbomethoxy-5-(*tert*-butyl)-pyrrole (VIII) gave consistently 16—17 % of "methoxy" instead of the calculated 12.1 %. We believe that the unexpectedly high values come from the splitting off of methyl groups as methyl iodide from the *tert*-butyl group. Huang and Morsingh[7] have reported another instance in which certain dimethylalkylaryl methanes behaved similarly.

2-(α-Hydroxyethyl)-furan[8] (IX) was electrolyzed in an ammonium bromide electrolyte[2] and the resulting dimethoxydihydrofuran X catalytically hydrogenated to the tetrahydrofuran XI.



2-(α-Acetoxy-β-acetamidoethyl)-furan (XII), prepared by catalytic hydrogenation of furfural cyanohydrin acetate[9] (XIII) in acetic anhydride, was also electrolyzed in an ammonium bromide electrolyte. The reaction product was not isolated pure, but hydrolyzed with sodium hydroxide to 2,5-dimethoxy-2-(α-hydroxy-β-aminoethyl)-2,5-dihydrofuran (XIV), which was obtained pure by distillation.



We consider that it now may be regarded as an established fact that alkoxylation of furans always takes place at the α-carbons. The structures of the new compounds X, XI and XIV are therefore proved by the syntheses.

EXPERIMENTAL

Microanalyses by E. Boss, K. Glens and W. Egger

2,5-Dimethoxy-2-carbomethoxy-5-isopropyl-2,5-dihydrofuran (III). 2-Carbomethoxy-5-*iso*propylfuran (I)[4] (10.5 g, b.p.$_{14}$ 106–107°, n_D^{25} 1.4834) was mixed with anhydrous methanol (45 ml) and concentrated sulfuric acid (0.375 g, 0.0038 mole) and the solution electrolyzed in the small cell described previously[10] (temperature of cooling-bath −20°).

Time hr	Current amp	Potential across the cell during electrolysis volt	Ampere hours (per cent of theoretical amount)
1.0	0.9	8.0	0.82 (25 %)
3.0	0.8	7.9	2.40 (72 %)
5.0	0.7	7.8	3.62 (108 %)
5.9	0.6	7.7	4.20 (125 %)

After electrolysis the yellow liquid was poured into a solution of sodium methoxide (from 175 mg of sodium (0.0076 mole)) in methanol (7 ml) and the methanol evaporated in a vacuum. Anhydrous ether (50 ml) was added to the oily, light-brown residue, a precipitate of sodium sulfate removed by filtration and the filtrate distilled in a vacuum.

Fraction (g)		B.p.$_{0.1}$ °C	n_D^{25}	OCH_3 Calc. 40.4 %
1	(1.8)	71	1.4500	37.8
2	(7.4)	71	1.4497	40.1
3	(3.3)	70–75	1.4502	41.0

The yield (all fractions) was 12.5 g (87 %, current efficiency 70 %) of III (colorless liquid). A portion of fraction 2 was also analyzed for carbon and hydrogen.

$C_8H_9O_2(OCH_3)_3$ (230.3) Calc. C 57.4 H 7.9
Found » 57.6 » 8.1

2,5-Dimethoxy-2-carbomethoxy-5-isopropyl-tetrahydrofuran (V). III (3.45 g) and anhydrous methanol (15 ml) were shaken (20 hr) with Raney nickel (0.5 g) under hydrogen (100 atm). The product was isolated by distillation. The yield was 3.10 g (89 %) of V (colorless liquid, b.p.$_{0.1}$ 66–67°, n_D^{25} 1.4426).

$C_8H_{11}O_2(OCH_3)_3$ (232.3) Calc. C 56.9 H 8.7 OCH_3 40.1
Found » 56.8 » 8.6 » 39.5

2-Carbomethoxy-5-(tert-butyl)-furan (II). This compound was prepared from methyl furoate (30.3 g, 0.24 mole), *tert*-butyl chloride (22.2 g, 0.24 mole) and aluminum chloride (48 g, 0.36 mole) after the directions of Gilman and Calloway[4]. From the final solution carbon disulfide and ether were removed by distillation through a Vigreux column and the residue distilled through a 5 cm column packed with Dixon gauze rings.

The yield was 37.6 g (86 %) of II (b.p.$_{11-12}$ 110—113°, n_D^{25} 1.4789); previously found[4] b.p.$_{15}$ 110—114° and n_D^{25} 1.4792. On standing the product crystallized (m.p. 27—28° (Hershberg apparatus, corr.)).

2,5-Dimethoxy-2-carbomethoxy-5-(tert-butyl)-2,5-dihydrofuran (IV). II (9.2 g) was electrolytically methoxylated as described above for the preparation of III. The yield was 12.0 g (97 %, current efficiency 78 %) of IV (colorless liquid, b.p.$_{0.2-0.3}$ 67—71°, n_D^{25} 1.4502).

$C_9H_{11}O_2(OCH_3)_3$ (244.3)	Calc.	C 59.0	H 8.3	OCH_3 38.1
	Found	» 59.1	» 8.3	» 37.6

2,5-Dimethoxy-2-carbomethoxy-5-(tert-butyl)-tetrahydrofuran (VI). IV (3.7 g) was hydrogenated and the reaction product isolated as described above for the preparation of V.

Fraction (g)	B.p.$_{0.1}$ °C	n_D^{25}	OCH_3 Calc. 37.8 %
1 (0.96)	70—74	1.4479	37.0
2 (2.55)	69—74	1.4473	37.4

The yield (both fractions) was 3.51 g (94 %) of VI (colorless liquid). A portion of fraction 2 was also analyzed for carbon and hydrogen.

$C_9H_{13}O_2(OCH_3)_3$ (246.3)	Calc.	C 58.5	H 9.0
	Found	» 58.7	» 8.9

1-Phenyl-2-carbomethoxy-5-(tert-butyl)-pyrrole (VIII). VI (2.46 g, 0.01 mole) was dissolved in a mixture of aniline (0.93 g, 0.01 mole) and glacial acetic acid (2.4 ml) and the solution heated under reflux for 5 minutes. The acetic acid and the methyl acetate formed by the reaction were removed by distillation in a vacuum. The yellow, crystalline residue was crystallized from ethanol-water, yielding 2.10 g (82 %) of VIII (slightly yellow crystals, m.p. 64—68°). Crystallization from ether-petroleum ether gave 1.46 g (57 %) of white crystals, m.p. 72°. Further crystallization did not change the m.p.

$C_{15}H_{16}ON(OCH_3)$ (257.3)	Calc.	C 74.7	H 7.4	N 5.4	OCH_3 12.1
	Found	» 74.7	» 7.8	» 5.0	"OCH_3" 16.9

The product gave a positive Ehrlich reaction after boiling with 15 % sulfuric acid for 2 minutes.

2,5-Dimethoxy-2-(α-hydroxyethyl)-2,5-dihydrofuran (X). 2-(α-Hydroxyethyl)-furan (IX)[8] (44.8 g, 0.40 mole) was electrolytically methoxylated and the reaction mixture worked up as described previously for the preparation of dimethoxydihydrofurfuryl alcohol[11].

Fraction (g)	B.p.$_{11-10}$ °C	n_D^{25}	OCH_3 Calc. 35.6 %
1 (7.0)	62—104	1.4641	17.0
2 (8.7)	104—107	1.4540	35.8
3 (42.0)	107	1.4542	35.6
4 (1.0)	107—108	1.4549	38.2
Residue (3)			

All fractions showed a negative Beilstein test for halogens. The yield (fractions 2 and 3) was 50.7 g (73 %, current efficiency 86 %) of X (colorless liquid). A portion of fraction 3 was also analyzed for carbon and hydrogen.

$C_6H_8O_2(OCH_3)_2$ (174.2)	Calc.	C	55.2	H	8.1
	Found	»	55.4	»	8.0

2,5-Dimethoxy-2-(α-hydroxyethyl)-tetrahydrofuran (XI). X (3.50 g) and anhydrous methanol (15 ml) were shaken (3 hr) with Raney nickel (0.50 g) under hydrogen (100 atm) and the reaction product isolated by distillation.

Fraction (g)	B.p.$_{15-16}$ °C	n_D^{25}	OCH_3 Calc. 35.2 %
1 (0.37)	102—104	1.4439	33.9
2 (2.06)	105—106	1.4456	34.8
3 (0.64)	107—108	1.4464	34.7

The yield (all fractions) was 3.07 g (87 %) of XI (colorless liquid). A portion of fraction 2 was also analyzed for carbon and hydrogen.

$C_6H_{10}O_2(OCH_3)_2$ (176.2)	Calc.	C	54.5	H	9.2
	Found	»	54.7	»	9.2

Hydrogenation for 20 hours did not change the yield or the quality of the reaction product.

Furfural cyanohydrin acetate (XIII). This compound was prepared from furfural (96 g) after the directions of Lukeš, Kastner, Gut and Herben[9]. The yield was 122 g (74 %) (b.p.$_{0.1}$ 67—68°, n_D^{25} 1.4683); previously found[9] b.p.$_{10}$ 115°. Two crystallizations from ether gave white crystals, m. p. 27—29°.

$C_6H_4O_2N(COCH_3)$ (165.1)	Calc.	C	58.2	H	4.3	N	8.5	$COCH_3$ 26.1
	Found	»	58.0	»	4.4	»	8.0	» 25.3

2,5-Dimethoxy-2-(α-hydroxy-β-aminoethyl)-2,5-dihydrofuran (XIV). XIII (33.0 g, 0.20 mole) and acetic anhydride (200 ml) were shaken (2 hr) with Raney nickel (4.0 g) under hydrogen (100 atm, 50—58°). After filtration the solvent was evaporated in a vacuum, finally under 0.1 mm at 55—60°. The residue was dissolved in methanol (250 ml), ammonium bromide (5.0 g) was added and the mixture electrolyzed with the set-up described previously[2] for the electrolytic methoxylation of furan.

Time hr	Current amp	Potential across the cell during electrolysis volt	Ampere hours (per cent of theoretical amount		Temperature in the cell °C
0.1	3.0	4.9	0.3	(3 %)	—13
0.5	2.9	4.8	1.5	(14 %)	—13
1.0	2.7	4.7	2.9	(27 %)	—14
3.5	2.3	5.3	9.2	(86 %)	—14
4.8	1.9	5.6	11.8	(110 %)	—14

After electrolysis the liquid was poured into a solution of sodium methoxide (from 5.8 g of sodium in methanol (70 ml)) and the methanol and the ammonia evaporated in a vacuum. Sodium hydroxide (3 *N*, 200 ml) was added, the mixture heated under reflux (20 hr), continuously extracted with ether and the etheral solution distilled. The yield was 14.5 g (38 % based upon furfural cyanohydrin acetate) of XIV (colorless liquid, b.p.$_{0.1}$ 91–93°, n_D^{25} 1.4836).

$C_4H_4O_2N(OCH_3)_3$ (189.2)	Calc.	C	50.8	H	8.0	N	7.4	OCH_3 32.8
	Found	»	50.9	»	8.2	»	7.6	» 32.3

In a separate experiment 2-(α-acetoxy-β-acetamidoethyl)-furan (XII) was isolated pure by crystallization from ether (white crystals, m. p. 64–67°).

$C_4H_7O_2N(COCH_3)_2$ (211.2)	Calc.	C	56.9	H	6.2	N	6.6	$COCH_3$ 40.8
	Found	»	56.8	»	6.1	»	6.5	» 39.8

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A New Synthesis of Pyridoxine

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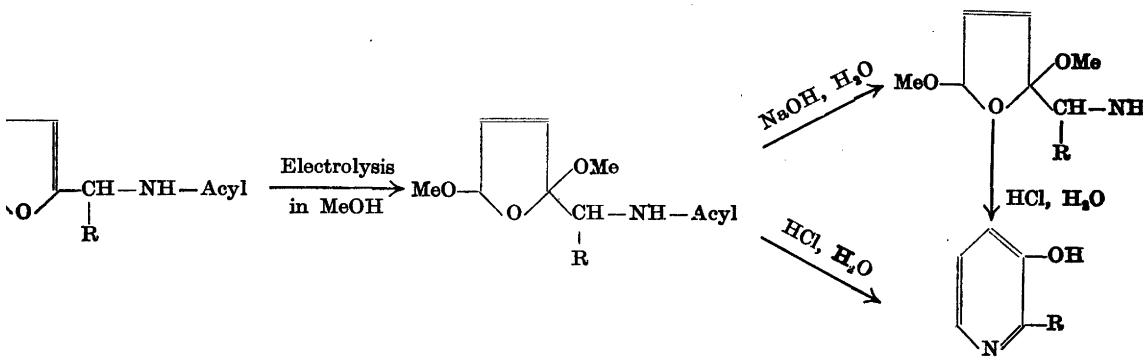
A new synthesis of pyridoxine from 2-(α-acetamidoethyl)-3,4-bis(acetoxymethyl)-furan is described.

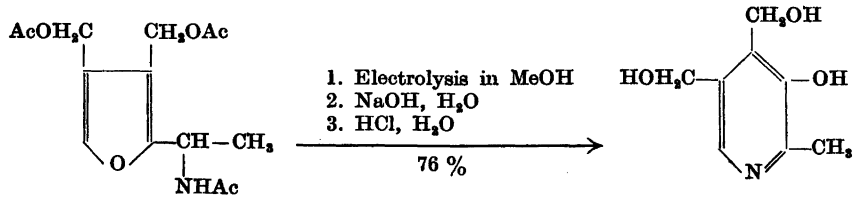
A new method for the preparation of 3-pyridols from N-acyl derivatives of 2-(α-aminoalkyl)-furans has recently been described[1]. The furan was first electrolytically methoxylated to the corresponding 2,5-dimethoxy-2,5-dihydrofuran and the latter was then heated under reflux with dilute hydrochloric acid to give the desired 3-pyridol. In order to obtain a good yield of the pyridol certain acyl derivatives required alkaline hydrolysis of the amide group prior to treatment with hydrochloric acid. The method was used for the preparation of 3-pyridol (I) and 2-methyl-3-pyridol (II) from various acyl derivatives of furfurylamine (III) and 2-(α-aminoethyl)-furan (IV).



III (R=H)
IV (R=CH_3)

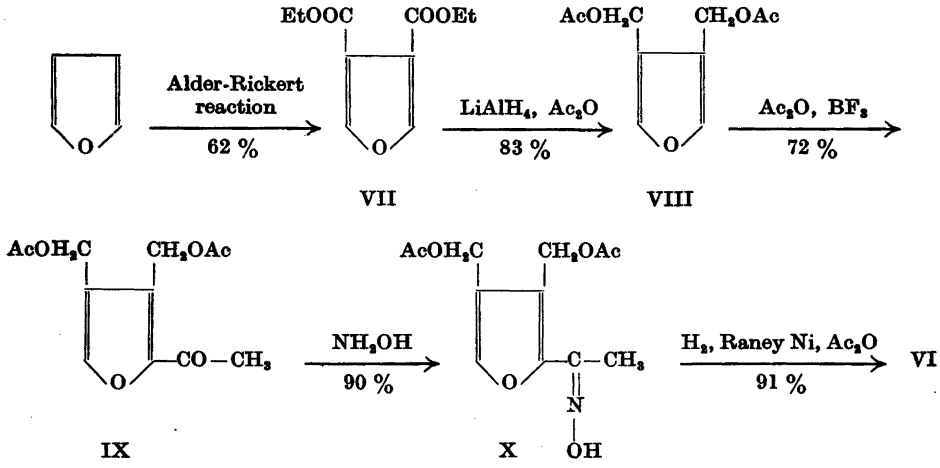
I (R=H)
II (R=CH_3)

We have used the above method for a synthesis of pyridoxine (2-methyl-4,5-bis(hydroxymethyl)-3-pyridol) (V) from 2-(α-acetamidoethyl)-3,4-bis(acetoxymethyl)-furan (VI). The overall yield of the three-step synthesis without isolation of the intermediate dimethoxydihydrofurans was 76 %.



VI V

VI was prepared from furan by the sequence of standard reactions shown below. The structure of compounds VI and VIII—X follows from their method of preparation and from analyses.



EXPERIMENTAL

Microanalyses by Ernst Boss and Kirsten Glens

3,4-Dicarbethoxyfuran (VII)[2]. Furan (13.0 g, 0.19 mole) and diethyl acetylenedicarboxylate (29.0 g, 0.17 mole) were mixed and heated (100°, 18 hr) under hydrogen (about 140 atm). After cooling acetone (120 ml) and 10 % palladium charcoal (0.22 g) were added and the mixture shaken (1 hr) under hydrogen (15—20 atm). The catalyst was removed by filtration and the filtrate distilled in a vacuum, whereby a pale-yellow liquid (28 g, b. $p._{18-15}$ 158—198°) was obtained. Redistillation through a short packed column gave 22.4 g (62 %) of VII (colorless liquid, b. $p._{13}$ 154—157°, n_D^{25} 1.4690, previously found[2] b. $p._{11}$ 140—165°). (Found: C 56.8; H 6.0; OC_2H_5 42.2. Calc. for $C_6H_2O_3(OC_2H_5)_2$ (212.2): C 56.6; H 5.7; OC_2H_5 42.5).

3,4-Bis(acetoxymethyl)-furan (VIII). 3,4-Dicarbethoxyfuran (3.18 g, 0.015 mole) in dry ether (15 ml) was added dropwise (10 min) to a stirred mixture of lithium aluminum hydride (0.69 g, 0.018 mole) and dry ether (20 ml). Stirring was continued for 5 minutes. Acetic anhydride (20 ml) was added during 5 minutes. The reaction flask was placed in an oil-bath and the temperature of the bath gradually raised to 110° whereby the ether distilled. The temperature of the bath was then maintained at 115—120° for 3 hours. After cooling, ether (40 ml) was added, the mixture filtered and the ether distilled. Distillation of the residue gave 2.63 g (83 %) of VIII (as a colorless liquid, which crystallized on cooling (b. p.$_{0.1}$ 88—90°, n_D^{25} 1.4672, m. p. 29—31° (Hershberg apparatus, corr.)). (Found: C 56.9; H 5.9; $COCH_3$ 40.0. Calc. for $C_6H_6O_3(COCH_3)_2$ (212.2): C 56.6; H 5.7; $COCH_3$ 40.6).
After crystallization from ether the m. p. was raised to 30—32°.

2-Acetyl-3,4-bis(acetoxymethyl)-furan (IX) [3]. VIII (22.0 g) was dissolved in acetic anhydride (25 ml) and a solution of boron fluoride etherate (1.3 ml) in acetic anhydride (20 ml) was added in one portion with stirring. The temperature rose to 40°. The mixture was heated to 85—90° and maintained at this temperature for 10 minutes. After cooling, water (40 ml) was added and stirring continued for 10 minutes. The dark-brown mixture was extracted with 80 ml and then with 50 ml of ether. The etheral extracts were washed with water, potassium hydrogen carbonate (2 *M*), water and dried. Distillation gave 18.9 g (72 %) of IX (almost colorless liquid, b. p.$_{0.1}$ 127—129°, m. p. 48—51°). (Found: C 56.9; H 6.0; $COCH_3$ 32.2. Calc. for $C_8H_8O_4(COCH_3)_2$ (254.2): C 56.7; H 5.6; $COCH_3$ 33.9).
After crystallization from ether the m. p. was raised to 51—53°.

2-Acetyl-3,4-bis(acetoxymethyl)-furan oxime (X). IX (18.5 g), anhydrous sodium acetate (8.2 g) and hydroxylamine hydrochloride (6.2 g) were mixed and heated under reflux (5 h) with ethanol (80 ml). The hot mixture was filtered and the ethanol evaporated in a vacuum. There remained an almost colorless liquid, which crystallized to a white solid after addition of water and cooling. Filtration, washing with water and drying gave 17.6 g (90 %) of X (m. p. 78—81°). (Found: C 53.6; H 5.9; N 5.4; $COCH_3$ 32.4. Calc. for $C_8H_9O_4N(COCH_3)_2$ (269.3): C 53.5; H 5.6; N 5.2; $COCH_3$ 32.0).
After crystallization from ether the m. p. was 79—81°.

2-(α-Acetamidoethyl)-3,4-bis(acetoxymethyl)-furan (VI). X (2.50 g) and acetic anhydride (20 ml) were shaken (1 hr) with Raney nickel (0.4 g) under hydrogen (100 atm, 70—80°). After filtration, the solvent was evaporated in a vacuum, finally under 0.1 mm at 60—70°. The residue was shaken with anhydrous ether (20 ml) whereupon white crystals were formed. About 10 ml of ether was distilled, the crystals were removed by filtration, washed twice with ether and dried. The yield was 2.52 g (91 %) of 2-(α-acetamidoethyl)-3,4-bis(acetoxymethyl)-furan (white crystals, m. p. 92—94°). (Found: C 56.3; H 6.4; N 4.5; $COCH_3$ 42.5. Calc. for $C_8H_{10}O_3N(COCH_3)_3$ (297.3): C 56.6; H 6.4; N 4.7; $COCH_3$ 43.4).
After crystallization from benzene-ether the m. p. was raised to 95—96°.

In another experiment the reaction product was isolated by distillation (yield 79 %, b. p. 170—190° at about 0.1 mm). The pale-yellow liquid crystallized after standing for some days. Crystallization of 500 mg from ether gave 350 mg of white crystals, m. p. 65—68°, which were readily soluble in ether. (Found: C 56.5; H 6.6; N 4.7; $COCH_3$ 42.4). Another crystallization from ether gave 280 mg, m. p. 66—68°.

An etheral solution of this product was seeded with the product from the first experiment (m. p. 95—96°) whereupon white crystals, m. p. 95—96°, which were slightly soluble in ether, were obtained.

VI may thus crystallize in two forms, of which the higher melting, slightly soluble form is apparently the one usually obtained.

Pyridoxine (V). VI (2.50 g) and ammonium bromide (300 mg) were dissolved in methanol (20 ml) and the solution electrolyzed with the set-up described previously [4] (temperature of cooling-bath −21°).

Time hr	Current amp	Potential across the cell during electrolysis volt	Ampere hours (per cent of theoretical amount)	
0.1	0.7	6.2	0.07	(16 %)
0.3	0.7	6.3	0.20	(44 %)
0.6	0.7	6.3	0.30	(67 %)
0.8	0.5	6.5	0.50	(110 %)

After electrolysis, the liquid in the cell was yellow. The liquid was poured into a solution of sodium methoxide (from 458 mg of sodium) in methanol (5 ml) and the methanol and the ammonia evaporated in a vacuum. Sodium hydroxide (3 *N*, 8.5 ml) was added, the mixture heated under reflux (21 hr) and then continuously extracted with ether (48 hr). The ether was evaporated in a vacuum and the residue heated under reflux (20 min) with hydrochloric acid (*N*, 15 ml). The mixture was evaporated to dryness in a vacuum and the residue was crystallized from 99 % ethanol. The yield was 1.19 g (69 %) of pyridoxine hydrochloride (white crystals, m. p. and mixed m. p. with authentic pyridoxine hydrochloride 207–209° (dec.)). The product was further characterized by the infrared absorption spectrum, which was identical over the whole range with that of pyridoxine hydrochloride. (Found: C 46.9; H 6.0; N 6.8; Cl 17.4. Calc. for $C_8H_{12}O_3NCl$ (205.6): C 46.7; H 5.9; N 6.8; Cl 17.2).

From the mother liquor a further 100 mg of pyridoxine hydrochloride (6 %) was obtained (almost white crystals, m. p. 206–208° (dec.)). (Found: C 46.8; H 5.9; N 6.8; Cl 17.3).

The extraction with ether was continued for 24 hours and the extract worked up as above. The yield was 20 mg (1 %) of pyridoxine hydrochloride (white crystals, m. p. 207–209° (dec.)). (Found: C 47.0; H 5.9; N 6.6; Cl 17.3).

The total yield of pyridoxine hydrochloride was thus 1.31 g (76 %).

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Preparation of Certain Methyl Polyhydroxybenzoates from Methyl Furoate

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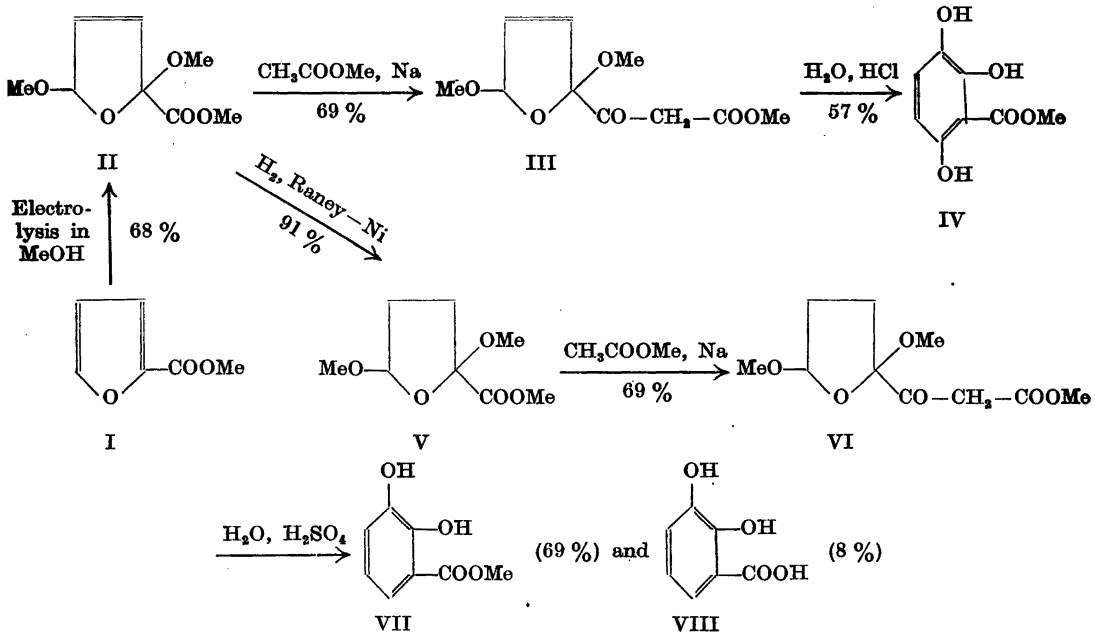
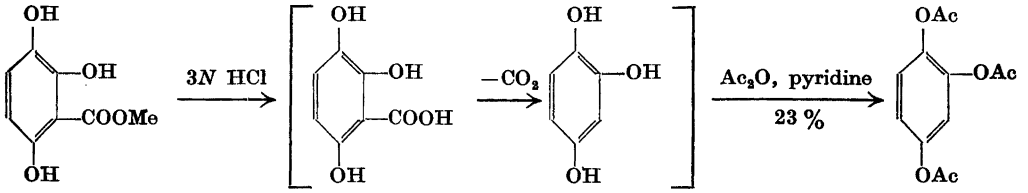
The methyl esters of 2,3-dihydroxybenzoic acid (VII) and 2,3,6-trihydroxybenzoic acid (IV) have been prepared from methyl furoate (I).

Furans with suitable substituents in the 2-position may be transformed into other systems with aromatic character by methoxylation to cyclic acetals of 1,4-dicarbonyl compounds, followed by intramolecular condensation in acid solution[1-4]. Further application of this principle has led to the preparation of the methyl esters of 2,3-dihydroxybenzoic acid (VII) and of 2,3,6-trihydroxybenzoic acid (IV) from methyl furoate (I) by the reactions shown below. A small amount of the free 2,3-dihydroxybenzoic acid (VIII) was also isolated.



Compounds III, IV and VI are new. Their structures follow from the syntheses and from analyses. The structure of IV was confirmed further by transformation into the known triacetate (X) of hydroxyhydroquinone (IX). With the preparation of the methyl ester of 2,3,6-trihydroxybenzoic acid (IV), all the six theoretically possible trihydroxybenzoic acids have been prepared, either as esters or as free acids.



IV IX X

EXPERIMENTAL

Microanalyses by Ernst Boss and Kirsten Glens

2,5-Dimethoxy-2,5-dihydro-2-furoyl acetic acid methyl ester (III) (cf.[5]*).* II[6] (51.7 g, 0.275 mole) is placed in a 0.5-liter three-necked flask fitted with a Hershberg stirrer, a dropping funnel and a reflux condenser and heated with stirring to 90°. 0.7 mm sodium wire (18.5 g, 0.81 mole) and methyl acetate (60.7 g, 0.82 mole) are added at 30—60 minutes intervals in six portions of equal size. During each addition of sodium the stirrer is stopped. As the reaction mixture becomes more and more viscous, benzene (70 ml in all) is added. After the reaction, methanol (30 ml) is added dropwise to remove residual sodium and the mixture left standing with stirring and heating over-night. The solution is cooled to −10° and poured in one portion with efficient stirring into a mixture of concentrated hydrochloric acid (90 g, 0.89 mole) and 170 g of cracked ice. The dark brown mixture is extracted rapidly with cold ether (500 + 100 + 100 ml) and the etheral solution washed with a 2 *M* solution of potassium hydrogen carbonate (300 ml) and dried with magnesium sulfate. The ether is removed by distillation and the residue distilled further under 0.1—0.2 mm from an oil bath (60—120°). After a fore-run of methyl acetoacetate, 43.4 g (69 %) of III is obtained [slightly yellow oil, b. p. 87—114° (main portion 104—114°), n_D^{25} 1.4588]. (Found: C 52.6; H 6.3; OCH_3 39.4. Calc. for $C_7H_5O_3(OCH_3)_3$ (230.2): C 52.2; H 6.1; OCH_3 40.4).

2,3,6-Trihydroxybenzoic acid methyl ester (IV). III (2.30 g) was shaken for 10 minutes with hydrochloric acid (0.1 *N*, 20 ml) under carbon dioxide. Hereby a homogeneous solution was obtained, which was left standing for 8 days. A light-violet precipitate was removed by filtration, washed with water and dried. The yield was 1.05 g (57 %) of IV [m. p. 131—135° (Hershberg apparatus, corr.)]. (Found: C 52.4; H 4.4; OCH_3 16.8. Calc. for $C_7H_5O_4(OCH_3)$ (184.1): C 52.2; H 4.4; OCH_3 16.9).

The product gave a reddish-brown ferric chloride reaction. After two crystallizations from methanol-water the m. p. was raised to 138°. Further crystallization did not change the m. p.

2,5-Dimethoxytetrahydro-2-furoyl acetic acid methyl ester (VI). This compound was prepared from 2,5-dimethoxy-2-carbomethoxy-tetrahydrofuran[6] (28.5 g) and methyl acetate (34.1 g) as described above for the preparation of III. The yield was 24.0 g (69 %) of VI (light-yellow liquid, b. $p._{0.1-0.2}$ 98—99°, n_D^{25} 1.4559). (Found: C 52.8; H 7.3; OCH_3 38.8. Calc. for $C_7H_7O_3(OCH_3)_3$ (232.2): C 51.7; H 6.9; OCH_3 40.1).

2,3-Dihydroxybenzoic acid methyl ester (VII) and 2,3-dihydroxybenzoic acid (VIII). VI (1.00 g) was heated under reflux (15 min) with sulfuric acid (0.1 *N*, 5 ml). After cooling, a precipitate was removed by filtration, washed with water and dried. The yield was 500 mg (69 %) of VII (almost white crystals, m. p. 73—74°). (Found: C 56.9; H 5.0; OCH_3 17.1. Calc. for $C_7H_6O_3(OCH_3)$ (168.1): C 57.1; H 4.8; OCH_3 18.5)

The product gave a blue ferric chloride reaction. After crystallization from benzene-petroleum ether the m. p. was raised to 77—78° (previously found [7] 78—79°).

The mother liquor was heated further under reflux (10 min) and continuously extracted with ether. Hereby 50 mg (8 %) of VIII was isolated (white crystals, m. p. 204—205°). (Found: C 54.7; H 4.1. Calc. for $C_7H_6O_4$ (154.1): C 54.6; H 3.9).

The product gave a violet ferric chloride reaction. After crystallization from benzene the m. p. was raised to 207° (previously found [8] 206°).

Hydroxyhydroquinone triacetate (X). IV (300 mg) was heated under reflux (3 hr) with hydrochloric acid (3 *N*, 10 ml) in an atmosphere of nitrogen. The solution was extracted with ether (15 + 15 ml), the etheral solution dried with magnesium sulfate and the ether distilled. The oily residue was heated under reflux (10 min) with a mixture of acetic anhydride (3 ml) and pyridine (3 ml). The reaction mixture was evaporated to dryness under 0.5 mm on a water-bath (60°) and the oily residue dissolved in ether (15 ml). The etheral solution was purified with carbon black and most of the ether removed by distillation. Hereby 95 mg (23 %) of X crystallized (white crystals, m. p. 86—88°). (Found: C 56.4; H 5.0; $COCH_3$ 51.0. Calc. for $C_6H_3O_3(COCH_3)_3$ (252.2): C 57.1; H 4.8; $COCH_3$ 51.2).

The infrared spectrum of the product was identical with that of an authentic sample. After crystallization from benzene-petroleum ether the m. p. was raised to 95° (previously found [9] 96—97°).

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ACTA CHEMICA SCANDINAVICA 9 (1955) 30—33

Some New Cyclic Hydroxamic Acids

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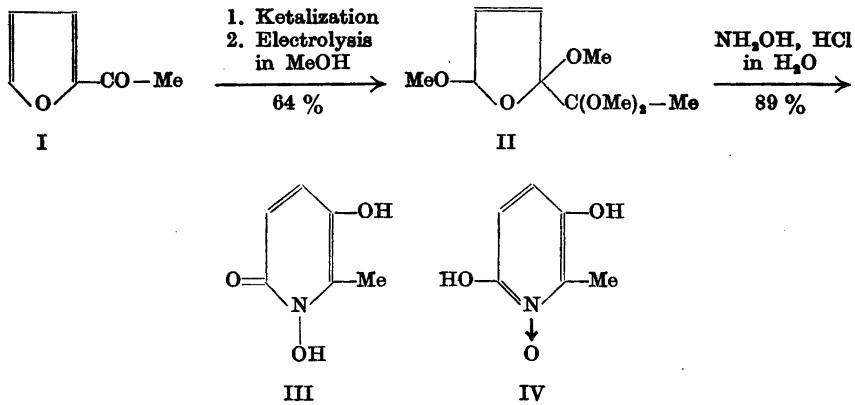
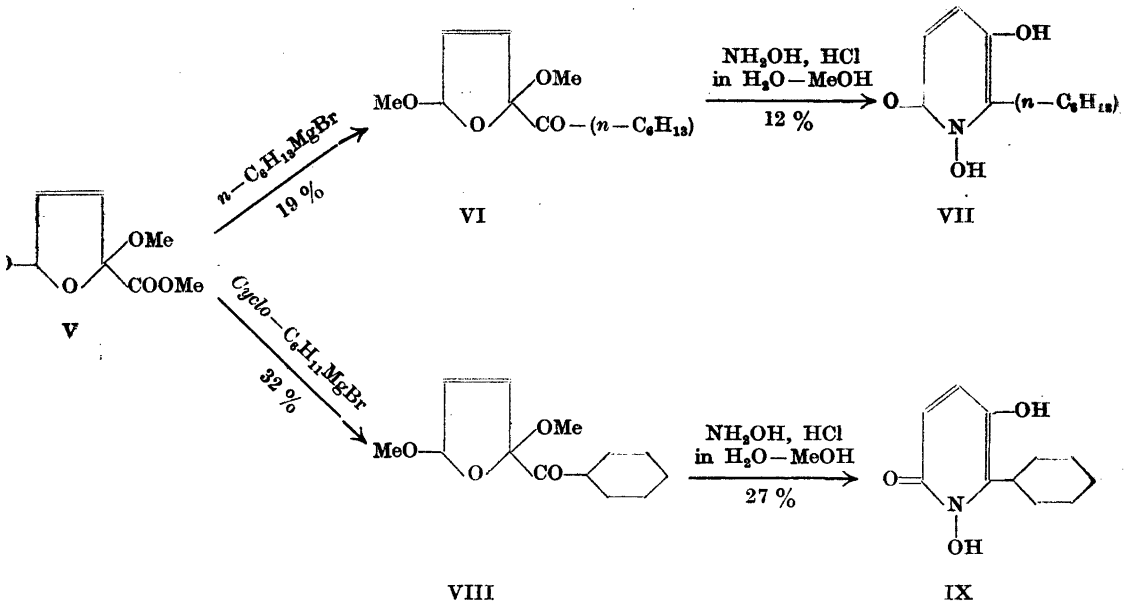
A compound prepared from 2-acetylfuran and previously [1] claimed by us to be 2-methyl-3,6-pyridinediol-1-oxide (IV) we now believe to be the tautomeric hydroxamic acid 1,5-dihydroxy-6-methyl-2-pyridone (III). Three similar cyclic hydroxamic acids have been prepared from methyl furoate (VII and IX) and furfural (XII), respectively.

We have recently found [1] that 2-acetylfuran (I) may be transformed by a two-step reaction into a compound, which was formulated as 2-methyl-3,6-pyridinediol-1-oxide (IV). Having become acquainted with experiments by Shaw, *et al.* [2-5], on certain cyclic hydroxamic acids related to the antibiotic aspergillic acid, we now think that our compound, rather than being a 2-hydroxypyridine-1-oxide, is the tautomeric hydroxamic acid 1,5-dihydroxy-6-methyl-2-pyridone (III).

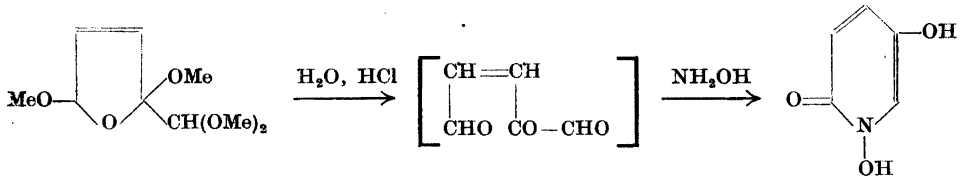


I II III IV

The new reaction has been used for the preparation of two more hydroxamic acids (VII and IX) by the reactions shown below. Compounds VI—IX are new. Their structures follow from the syntheses and from analyses.

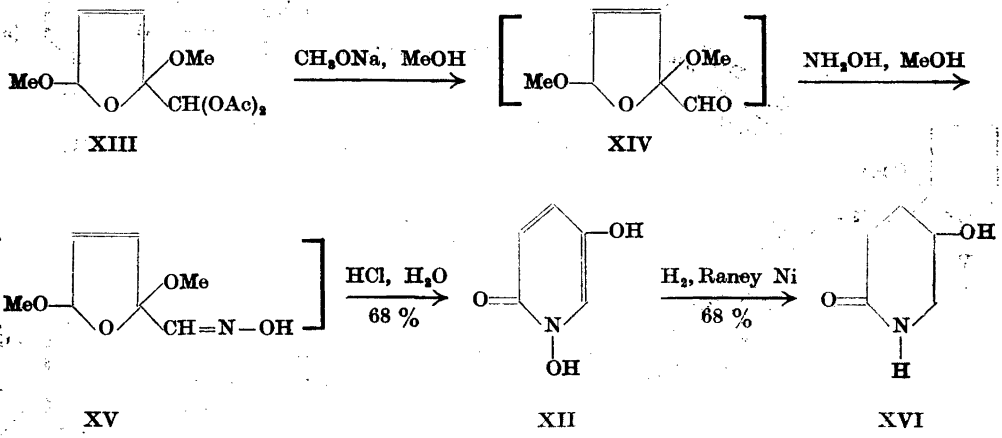


The corresponding unsubstituted hydroxamic acid (1,5-dihydroxy-2-pyridone) (XII) has also been prepared though under somewhat different reaction conditions. One might expect any easily hydrolyzable derivative of 2-*cis*-pentene-1,5-dial-4-one (XI) to react with hydroxylamine hydrochloride to give (XII). 2,5-Dimethoxy-2-dimethoxymethyl-2,5-dihydrofuran [6] (X) gave, however, only a dark reaction mixture, from which no XII could be isolated. The mixture nevertheless gave a strong violet ferric chloride reaction indicating that a small amount of XII actually was formed.



A good yield of XII was obtained by using 2,5-dimethoxy-2,5-dihydrofurfural [7] (XIV) as a starting material. A methanolic solution of this compound was prepared from 2,5-dimethoxy-2-diacetoxymethyl-2,5-dihydrofuran (XIII), the solution treated with hydroxylamine and the reaction product hydrolyzed and condensed to XII. Probably the reaction proceeds through the oxime XV or its equivalent, the initial attachment of the hydroxylamine mole-

cule to the organic molecule thus taking place at one of the ends of the chain of carbon atoms as required for the formation of XII.



XIII XIV XV XII XVI

The structure of the new hydroxamic acid XII was proved by catalytic hydrogenation to the known 5-hydroxy-2-piperidone XVI.

EXPERIMENTAL

Microanalyses by E. Boss and K. Glens

2,5-Dimethoxy-2-(α-oxo-n-heptyl)-2,5-dihydrofuran (VI). An etheral solution of *n*-hexylmagnesium bromide [from *n*-hexyl bromide (36.0 g, 0.22 mole) and 70 ml of ether] was added at −60° to a solution of V[8] (37.6 g, 0.20 mole) in 150 ml of ether. A saturated solution of ammonium chloride (32 ml) was added at 5° and the etheral solution removed by decantation. The residue was extracted twice with 35 ml of ether. Distillation of the combined etheral solutions through a 5 cm column [9] first gave a forerun (13 g, b.p.$_{17}$ 68–157°) consisting mainly of V, and then 9.1 g (19 %) of VI (almost colorless liquid, b.p.$_{17}$ 157–159°, n_D^{25} 1.4483). (Found: C 64.6; H 9.0; OCH_3 25.6. Calc. for $C_{11}H_{16}O_2(OCH_3)_2$ (242.3): C. 64.4; H 9.2; OCH_3 25.6).

About 15 ml of a higher boiling residue, consisting mainly of the corresponding tertiary alcohol, remained in the distillation flask.

1,5-Dihydroxy-6-(n-hexyl)-2-pyridone (VII). VI (1.21 g, 0.0050 mole) was dissolved in methanol (3 ml) and the solution added to a solution of hydroxylamine hydrochloride (0.44 g, 0.0063 mole) in methanol (3 ml) — water (2.5 ml). After standing (17 hr) the light-brown reaction mixture was evaporated to dryness in a vacuum from a water bath (25°). The residue was dissolved in ammonium hydroxide (20 %, 4.0 ml) and continuously extracted with ether. The etheral solution was evaporated to dryness and the residue crystallized from ether. The yield was 126 mg (12 %) of VII [almost white crystals, m.p. 86–88° (Hershberg app., corr)]. The crystals gave a strong violet ferric chloride reaction. (Found: C 62.8; H 8.4; N 6.5. Calc. for $C_{11}H_{17}O_3N$ (211.3): C 62.5; H 8.1; N 6.6).

2,5-Dimethoxy-2-[α-oxo-α-(cyclohexyl)-methyl]-2,5-dihydrofuran (VIII). This compound was prepared from cyclohexyl bromide (24.0 g) and V (25.0 g) as described for the preparation of VI. The yield was 10.2 g (32 %) of VIII (almost colorless liquid, b.p.$_{14}$ 160–162°, n_D^{25} 1.4732). (Found: C 64.8; H 8.2; OCH_3 25.8. Calc. for $C_{11}H_{14}O_2(OCH_3)_2$ (240.3): C 65.0; H 8.4; OCH_3 25.8).

1,5-Dihydroxy-6-cyclohexyl-2-pyridone (IX). This compound was prepared from VIII (1.20 g) as described for the preparation of VII. The crude product was crystallized from methanol-ether. The yield was 286 mg (27 %) of IX (almost white crystals, m.p. 180—182°). The crystals gave a strong violet ferric chloride reaction. (Found: C 62.9; H 7.4; N 6.5. Calc. for $C_{11}H_{15}O_3N$ (209.2): C 63.1; H 7.2; N 6.7).

1,5-Dihydroxy-2-pyridone (XII). XIII (500 mg, 0.0019 mole) was dissolved in methanol (2.0 ml) and the solution added to a solution of sodium methoxide [from 44 mg of sodium (0.0019 mole) in methanol (1.0 ml)]. After standing for 15 minutes a solution of hydroxylamine hydrochloride (0.16 g, 0.0023 mole) and anhydrous sodium acetate (0.050 g) in methanol (2.0 ml) was added, the mixture left standing for 20 minutes and a precipitate of sodium chloride removed by filtration. The almost colorless filtrate was heated under reflux (8 min) and then evaporated in a vacuum from a water bath (30—40°) to about 1 ml. Water (1 ml) and then *N* hydrochloric acid (2.5 ml) were added and the solution left standing for 10 minutes. A precipitate of white crystals of XII was removed by filtration and washed with water, ethanol and ether. The yield was 164 mg (68 %); m.p. in an evacuated tube 216—225° (dec.). Crystallization from ethanol gave 138 mg (57 %), m.p. 215—223°. The product gave a strong violet ferric chloride reaction. (Found: C 47.3; H 4.2; N 10.8. Calc. for $C_5H_5O_3N$ (127.1): C 47.3; H 4.0; N 11.0).

In a similar experiment starting from 2,5-dimethoxy-2,5-dihydrofurfural (XIV) (600 mg) practically the same yield of XII (330 mg = 69 %) was obtained. (Found: C 46.9; H 4.2; N 10.8).

In an attempt to prepare XII from 2,5-dimethoxy-2-dimethoxymethyl-2,5-dihydrofuran (X), X (0.41 g) and hydroxylamine hydrochloride (0.21 g) were dissolved in water (7 ml) and the solution left standing over-night. The next day the dark reaction mixture gave a strong violet ferric chloride reaction but isolation of pure XII failed.

5-Hydroxy-2-piperidone (XVI). XII (0.47 g) and anhydrous methanol (25 ml) were shaken (1 hr) with Raney nickel (0.2 g) under hydrogen (100 atm., 100°). The Raney nickel was removed by filtration and the filtrate evaporated in a vacuum. The crystalline residue was crystallized from methanol-ether. The yield was 0.29 g (68 %) of XVI (almost colorless crystals, m.p. 144—146°, previously found 145—146° [10, 11]). (Found: C 51.9; H 7.7; N 12.0. Calc. for $C_5H_9O_2N$ (115.1): C 52.2; H 7.9; N 12.2.)

Recrystallization from methanol-ether did not change the m.p.

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Studies on the 7α-Hydroxylation of Taurodesoxycholic Acid in Rat Liver Homogenates. Bile Acids and Steroids 18

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Fractionation of rat liver homogenate into a particle free supernatant (1 hr, 100 000 × g) and a crude residue containing microsomes, mitochondria *etc.* has been carried out. It is shown that the particle-free medium is capable of hydroxylating taurodesoxycholic acid in the 7 α-position yielding taurocholic acid. About 40 % of the amount hydroxylated in total homogenates is hydroxylated by supernatant alone.

The addition of ATP greatly stimulates the hydroxylation. Supernatant from 1 g liver can hydroxylate almost 1 mg of taurodesoxycholic acid in 2 hours. Versene increases the yield at a certain concentration range but inhibits in greater concentrations.

The conversion of desoxycholic acid into taurocholic acid has been demonstrated to occur in the rat *in vivo* [1] as well as *in vitro* in liver slices [2] and homogenates [3,4,5]. We are now presenting some results of experiments on the 7α-hydroxylation of taurodesoxycholic acid-24-^{14}C in rat liver homogenates. These homogenates have also been fractionated and the effect of different additions on the hydroxylation has been investigated.

EXPERIMENTAL

Taurodesoxycholic acid-24-^{14}C was prepared according to Bergström and Norman [6] from desoxycholic acid-24-^{14}C[7]. We are indebted tc Dr. A. Norman and fil.kand. K. Pääbo, respectively, for preparing these compounds.

The method used for paper chromatography of free and conjugated bile acids has been described by Sjövall [8] and the measurement of the radioactivity of the paper strips has been described earlier [3]. The homogenizing technique used has been described earlier but we have now used the medium proposed by Bucher [9,10] with only minor changes. One litre of the medium contained 10.8 g of monobasic potassium phosphate, 3.2 g of potassium hydroxide, 1 g of magnesium chloride (6 H_2O) and 3.6 g of nicotinic acid amide. The pH was 7.5 ± 0.1. The incubation vessel, a small pyrex test tube containing 1 ml of homogenate or homogenate fraction with the additions indicated was shaken in a water bath at 37° for 2 hours. The reaction was stopped by the addition of five volumes of ethanol and the resulting mixture was filtered and worked up as described earlier [3].

The crude homogenates were centrifuged in a precooled tube of an ordinary centrifuge at 400 × g for 10 minutes, yielding what is referred to as "total homogenate" in the following. To prepare a particle-free supernatant of this homogenate it was centrifuged at 100 000 × g for 60 min. in a preparative Spinco ultracentrifuge. The fatty top layer was then carefully pipetted off and the rest of the supernatant used for the incubations. In some experiments the whole sediment (mitochondria, microsomes, "fluffy" layer) was stirred up in the appropriate amount of homogenizing medium and used without further differentiation and is referred to as "crude residue". The supernatant contained about 2 mg of nitrogen per ml (corrected for the content of nicotinamide) whereas the total homogenate contained about 3.4 mg of N per ml.

RESULTS

The total homogenate was first separated into sediment and particle-free supernatant at 100 000 × g. The 7α-hydroxylation of taurodesoxycholate to taurocholate and the conjugation of cholic acid with taurine was then investigated with the supernatant and with the resuspended sediment.

The results of 4 representative experiments of each type are shown in Tables 1 and 2.

Table 1. 7α-hydroxylation of 50 μg taurodesoxycholic acid-24-^{14}C in rat liver homogenate and fractions thereof. 1 ml incubation medium corresponding to about 200 mg rat liver fresh weight. Incubation: 2 hours; pH 7.5; air.

Expt. No.	Hydroxylated amount in μg			
	total homogenate	supernatant	total sediment	supernatant + total sediment
1	22	16	8	22
2	13	6	9	26
3	35	9	7	41
4	38	12	6	24
Average values	27	10.8	7.5	28

Table 2. Conjugation of cholic acid with taurine in rat liver homogenate and fractions thereof. 1 ml incubation medium corresponding to about 200 mg of liver fresh weight. Each vessel contained 50 μg of cholic acid, no other additions. 2 hrs 37° in air, pH = 7.5.

Expt. No.	Taurocholic acid formed in μg			
	total homogenates	supernatant	total sediment	supernatant + total sediment
1	39	7	7	29
2	38	6	9	38
3	38	6	7	41
4	41	6	3	42
Average values	39	6.3	6.5	38

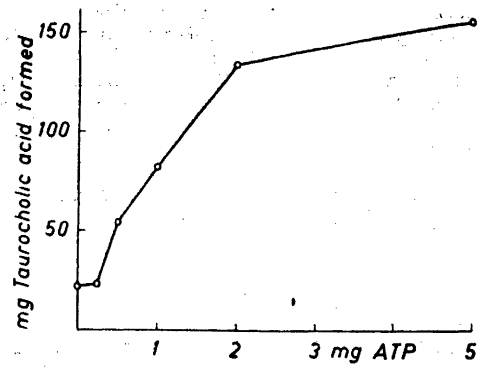

Fig. 1. Effect of increasing amounts of ATP on 7 α-hydroxylation of taurodesoxycholate-24-^{14}C. (0.2 mg). Reaction time: 2 hours at 37°, air.

These results show that the supernatant without any additions has approximately 40 % of the hydroxylating capacity of the total homogenate whereas the conjugation only occurred to about 15 % of that of the total homogenate or of the recombined fractions. Further data on the conjugation of bile acids in fractionated liver homogenates are being published by Bremer [18].

The effect of adenosinetriphosphate (ATP) on hydroxylation is illustrated in Fig. 1. In this experiment one ml of the supernatant hydroxylated about 22 μg taurodesoxycholate whereas this amount increased to a maximum of 134 μg after addition of 2 mg of ATP.

The effect of ATP on the hydroxylation was more marked in the supernatant than in the total homogenate where presumably the structural element contains both energy yielding and consuming systems. In one experiment similar to that shown in Fig. 1, 5 mg of ATP caused a seven fold increase to

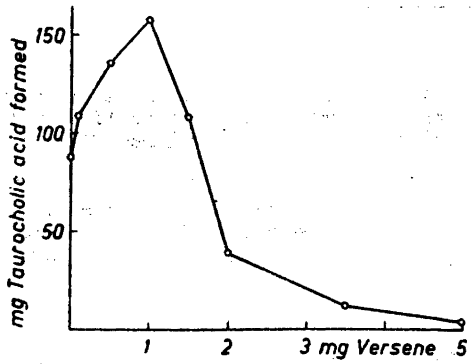

Fig. 2. Effect of versene on hydroxylation of taurodesoxycholate. Each vessel: Rat liver supernatant (1 ml) + taurodesoxycholic acid-24-^{14}C + ATP (1 mg). Incubation 2 hours at 37° in air.

Table 3. Influence of ATP, DPN and fumarate on 7 α-hydroxylation of taurodesoxycholate (50 μg) in 1 ml of supernatant. Incubation at 37° in air for 2 hours.

	μg taurocholic acid found
Total homogenates	19
Supernatant	17
» + 2 μmoles ATP	37
» + 1 μmole DPN	15
» + 1 μmole fumarate	14
» + DPN + fumarate	13
» + ATP + DPN	36
» + ATP + fumarate	35
» + ATP + DPN + fumarate	34

188 μg of hydroxylated product in 1 ml of the supernatant whereas the total homogenate only reached 92 μg with the same addition.

We found earlier that the hydroxylating system is very sensitive to some heavy metal ions, and have therefore investigated the influence of a chelating agent, versene, *i. e.* ethylenediamine tetraacetic acid.

A typical experiment is shown in Fig. 2 in which increasing amounts of versene were added to 1 ml of the supernatant to which, furthermore, 2 mg of ATP had been added.

There was almost a doubling of the yield after the addition of 1 mg (3 μmoles) of versene but larger amounts rapidly caused an almost complete inhibition. Sweat [13] reported some inhibition of the 11 α-hydroxylase with versene.

The effect of fumarate and diphosphopyridine nucleotide (DPN) either alone or in combination with ATP is shown in Table 3. From these results it can be concluded that in the present system ATP is the only compound showing any noticeable effect. Some experiments with addition of TPN, reduced DPN and cytochrome c did not cause any noticeable changes in the results. Work is now in progress to fractionate the supernatant in order to facilitate further studies of the enzymic mechanisms involved.

The 7 α-hydroxylase system studied is thus contained mainly in the particle-free supernatant and the activity is greatly stimulated by ATP.

This is thus at variance with the 11 α-hydroxylase [12-13] from adrenals that has been shown to be contained in the particles isolated between 2 000 × g and 19 000 × g [13] or at 5 000 × g [14] whereas the supernatant has been found to be practically inactive in this respect.

The 17α- and 21-hydroxylases are, however, apparently contained mainly in the supernatant (*cf.*[14], page 175, footnote 2).

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ACTA CHEMICA SCANDINAVICA 9 (1955) 39—48

The Complex Formation between Iron(III)Ion and Some Phenols. III. Salicylaldehyde, *o*-Hydroxyacetophenone, Salicylamide and Methyl salicylate

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The complex formation between iron(III)ion and the following phenolic substances has been investigated: salicylaldehyde, *o*-hydroxyacetophenone, salicylamide and methyl salicylate. All measurements have been made at 25.0° ± 0.1° C and in solutions with a concentration of ClO_4^- of 3 000 mC. By potentiometric and photometric methods the complexity constants, defined as follows have been determined

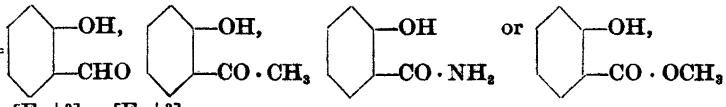
$$\varkappa_n = \frac{[H^+]^n[FeR_n^{3-n}]}{[RH]^n[Fe^{+3}]} \quad (n = 1 \text{ or } 2)$$

RH is the phenolic substance concerned. Further the molar extinctions (1 000 ε_n) have been determined. The results are listed in Table 12.

In the previous works in this series the complex formation between iron(III)-ion and some chelating phenols have been studied, namely sulfosalicylic acid [1], salicylic acid and *p*-aminosalicylic acid [2]. In this work the complex formation with salicylaldehyde, *o*-hydroxyacetophenone, salicylamide and methyl salicylate is investigated. All these substances may give chelate complexes with iron(III)ion.

Symbols used in the text (mC denotes millimoles/liter).

h = hydrogen ion concentration, mC.
[] = concentration of substance indicated, mC.
$[]_t$ = stoichiometrical concentration, mC.

RH = 

$$b = \frac{[Fe^{+3}]_t - [Fe^{+3}]}{[Fe^{+3}]}$$

ε = extinction, $cm^{-1}mC^{-1}$
E = extinction, cm^{-1}
$\varkappa_n$ = complexity constant
$\varkappa_{nm}$ = complexity constants of the hydroxyl complexes $Fe_n(OH)_m$

$$\varkappa' = \frac{\varkappa_{11}}{h} + \frac{\varkappa_{12}}{h^2} + \frac{2\varkappa_{22}[Fe^{+3}]}{h^2}$$

The signs for the electrical charges are omitted when there is no risk of confusion.

The complexes are formed according to the following equation

$$Fe + n\ RH = n\ H + FeR_n$$

and the complexity constants are defined as follows:

$$\varkappa_n = \frac{h^n[FeR_n]}{[RH]^n[Fe]} \quad (n = 1,\ 2\ldots) \tag{1}$$

The same equations are valid whether or not chelates are formed. In either case only one hydrogen ion is set free per RH molecule.

In determining the complexity constants the same methods have been used as in the previous works [1,2]. However, in this investigation some complications are met with. The substances studied are only slightly soluble (5—15 mC) in the medium used, 3 000 mC $NaClO_4$, at 25° C. Due to this only the lowest complexes are formed, FeR and perhaps FeR_2. More than two molecules R per atom Fe have not been found. Due to the low complex forming tendency and the low concentration of R the hydroxyl complexes of iron(III)ion have been formed in higher amounts, appreciably influencing the calculations.

In the potentiometric determinations the following equation, derived before, has been used:

$$\frac{(b-\varkappa')h}{[RH]} = \varkappa_1 + \varkappa_2 \frac{[RH]}{h} + \ldots \tag{2}$$

$\varkappa'$, which in the earlier investigations was a small correction term, is now of the same order as b. This causes uncertainties in the results.

In the photometric determinations the following equations, also derived before, have been used:

$$\frac{c}{E} = \frac{1}{\varepsilon_1} + \frac{h}{[RH]} \frac{1}{\varkappa_1\varepsilon_1} \tag{3}$$

which is valid when $[Fe]_t = [R]_t = c$, and when only FeR is formed in measurable amount.

$$\frac{[Fe]_t}{E} = \frac{1}{\varepsilon_1} + \frac{h}{[RH]} \frac{1}{\varepsilon_1\varkappa_1} \tag{4}$$

$$\frac{[Fe]_t}{E} = \frac{1}{\varepsilon_2} + \frac{h}{[RH]} \frac{E-[Fe]_t\varepsilon_1}{E} \frac{\varkappa_1}{\varepsilon_2\varkappa_2} \tag{5}$$

Equations (4) and (5) are valid when $[R]_t \gg [Fe]_t$. Equ. (4) is used in a range where only FeR is formed in measurable amount. Equ. (5) is used when FeR and FeR_2 are formed, but no other forms of Fe are present.

Equations (3)—(5) were derived assuming that no appreciable amounts of hydroxyl complexes are formed.

Apparatus and chemicals. The same apparatus has been used as in previous investigations [1].

The preparation and quantitative determination of $Fe(ClO_4)_3$, $Fe(ClO_4)_3$ and $NaClO_4$ have been described earlier.

Salicylaldehyde was purified as the bisulfite compound and by freezing out. *o*-Hydroxyacetophenone was made according to Rosenmund and Schnurr [3] by heating phenyl

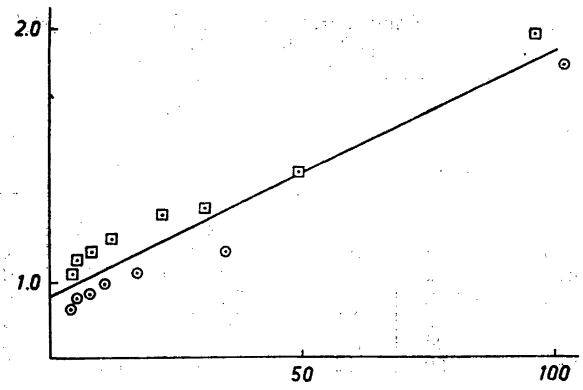


Fig. 1. Salicylaldehyde. Potentiometric determination of $\varkappa_1$ *and* $\varkappa_2$. $\frac{(b-\varkappa')h}{[RH]}$ *vs.* $\frac{[RH]_t}{h}$.
○ : Fe(III) = 0.1051 mC, □ : Fe(III) = 0.0496 mC.

acetate and water-free $AlCl_3$. The raw product was purified by steam distillation. This product was colourless. The salicylamide used had a melting point 138–139° C.

The quality of the methyl salicylate used was controlled according to the Swedish Pharmacopeia Ed. XI. It contained no free acid.

All the solutions were made up by weight, and $NaClO_4$ added to a total concentration of 3 000 mC ClO_4^-. Under these conditions it is assumed that the activity factors are constant and hence the concentrations may be used instead of activities in the equilibria.

SALICYLALDEHYDE

The complex formation between iron(III)ion and salicylaldehyde has been studied by Herbst and coworkers [4] and by Broumand and Smith [5]. They have shown, using the method of continuous variations [6] that the complex is built up from one molecule of salicylaldehyde per atom Fe. The complex formation between salicylaldehyde and bivalent metals has been thoroughly investigated

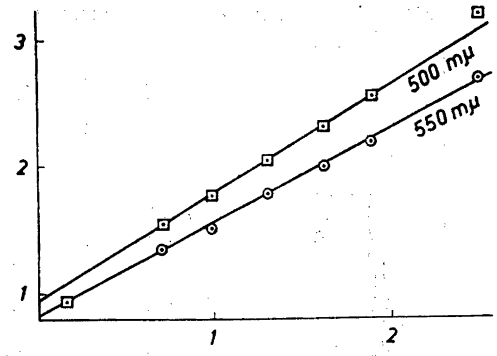


Fig. 2. Salicylaldehyde. Photometric determination of $\varkappa_1$. $[Fe]_t/E$ *vs.* $h/[RH]$.
$[R]_t$ = 6.664 mC, $[Fe]_t$ = 0.696 mC.

Table 1. Salicylaldehyde. Photometric determination of $\varkappa_1$. $[Fe]_t = 0.696$ mC; $[R]_t = 6.664$ mC

h	Extinction at wave length						
	400	450	500	550	600	650	700
1.012	0.828	0.562	0.740	0.845	0.661	0.422	0.242
4.708	0.500	0.327	0.452	0.513	0.419	0.265	0.155
6.604	0.461	0.300	0.392	0.458	0.363	0.237	0.133
8.740	0.398	0.252	0.337	0.390	0.316	0.203	0.112
10.82	0.345	0.219	0.300	0.347	0.270	0.174	0.100
12.65	0.321	0.204	0.272	0.317	0.256	0.160	0.090
16.67	0.263	0.160	0.218	0.259	0.208	0.131	0.072

and it is generally assumed that a chelate is formed. Some of these metals form complexes with 1 or 2 molecules of aldehyde per metal atom.

In the potentiometric investigation of this complex formation, this author has shown that complexes with 1 and 2 molecules per atom Fe are formed. However, the higher complex has such a low stability that it was impossible to make a photometric investigation because only a very small fraction of the complex-bound iron was contained in the form of the complex FeR_2 even when [RH]/h was made as high as possible.

Salicylaldehyde is slightly soluble (*ca.* 10 mC) in 3 000 mC $NaClO_4$ at 25° C. Thus, it was impossible to work with high concentrations of iron without the OH-complexes becoming dominant. Only two concentrations of Fe(III) have been studied in the potentiometric measurements. The results of these experiments are shown in Fig. 1. From these curves the values of $\varkappa_1$ and $\varkappa_2$ are calculated and given in Table 12.

The photometric determinations are shown in Table 1 and Fig. 2. This figure shows the curves at two wave lengths, the values being calculated with equ. (3). The results are given in Table 12.

Table 2. o-Hydroxyacetophenone. Potentiometric determination of $\varkappa_1$. $[Fe]_t = 0.1051$ mC. Result: $\varkappa_1 = 0.43$ (mean value).

h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')\,h}{[RH]}$
1.646	4.0881	7.787	0.429
1.258	5.4517	10.165	0.437
0.9656	7.5052	13.218	0.455
0.6776	10.886	18.803	0.449
0.4085	18.260	31.141	0.419
0.3286	23.998	38.691	0.430
0.2482	33.129	51.201	0.418
0.1882	48.591	67.487	0.442
0.1321	84.516	96.101	0.515
0.1079	149.95	117.60	0.845

Table 3. o-Hydroxyacetophenone. Potentiometric determination of $\varkappa_1$. $[Fe]_t = 0.0496$ mC. Result: $\varkappa_1 = 0.70$ (mean value).

h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')h}{[RH]}$
1.225	8.2296	10.545	0.679
0.8794	11.670	14.660	0.682
0.5843	17.962	22.026	0.680
0.3469	32.083	37.042	0.685
0.2158	57.170	59.500	0.680
0.1776	72.758	72.280	0.721
0.1122	136.49	114.349	0.783

Table 4. o-Hydroxyacetophenone. Photometric determination of $\varkappa_1$. $[R]_t = 11.91$ mC; $[Fe]_t = 0.696$ mC.

h	Extinction at wave length						
	400	450	500	550	600	650	700
1.069	0.705	0.480	0.708	0.803	0.615	0.390	0.225
3.021	0.555	0.364	0.541	0.620	0.500	0.318	0.180
5.089	0.470	0.294	0.450	0.504	0.412	0.254	0.147
7.166	0.400	0.248	0.378	0.436	0.346	0.211	0.120
9.086	0.359	0.215	0.335	0.380	0.300	0.184	0.110
11.13	0.318	0.200	0.300	0.340	0.270	0.172	0.096
13.00	0.288	0.176	0.266	0.303	0.235	0.146	0.084

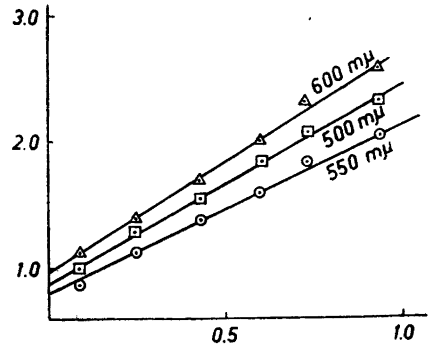


Fig. 3. o-Hydroxyacetophenone. Photometric determination of $\varkappa_1$. $[Fe]_t/E$ *vs.* h/[RH]. $[R]_t = 11.91$ mC, $[Fe]_t = 0.696$ mC.

o-HYDROXYACETOPHENONE

Recently, Jatkar and Mattoo [11] have published a work where they show the formation of complexes with one molecule of *o*-hydroxyacetophenone per atom Fe. They have determined the complexity constant $K_1 = [Fe][R]/[FeR] = 14 \cdot 10^{-4}$ C, where they assume [R] = [RH]. However, this value of the constant is of little interest as no pH measurements seem to have been made.

Table 5. Salicylamide. Potentiometric determination of $\varkappa_1$ and $\varkappa_2$. $[Fe]_t = 0.484$ mC. Results: $\varkappa_1 = 13.7$; $\varkappa_2 = 0.21$.

h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')h}{[RH]}$
0.9107	50.961	3.413	14.46
0.7211	64.887	4.298	14.58
0.5556	85.520	5.558	14.80
0.4150	117.59	7.405	15.20
0.3607	136.49	8.514	15.29
0.3209	157.17	9.520	15.71
0.2757	185.98	11.055	15.94
0.2415	218.33	12.555	16.41
0.2028	269.63	14.911	16.98

Table 6. Salicylamide. Potentiometric determination of $\varkappa_1$ and $\varkappa_2$. $[Fe]_t = 0.484$ mC. Results: $\varkappa_1 = 13.0$; $\varkappa_2 = 0.53$.

h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')h}{[RH]}$
1.595	26.767	1.807	14.38
1.200	36.326	2.398	14.69
0.7918	56.719	3.665	14.94
0.4849	96.996	5.865	15.85
0.2434	224.39	11.27	18.84
0.1342	509.40	20.06	23.69
0.1079	729.17	24.42	27.79

Table 7. Salicylamide. Potentiometric determination of $\varkappa_1$ and $\varkappa_2$. $[Fe]_t = 0.1051$ mC. Results: $\varkappa_1 = 14.0$; $\varkappa_2 = 0.32$.

h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')h}{[RH]}$
1.339	39.034	2.633	14.46
0.9845	54.732	3.578	14.90
0.7073	79.666	4.989	15.52
0.4556	131.75	7.733	16.47
0.2473	277.11	14.185	18.71
0.08714	1 141.4	39.706	26.86
0.04208	3 628.3	81.535	40.84
0.02421	9 307.7	140.975	59.83

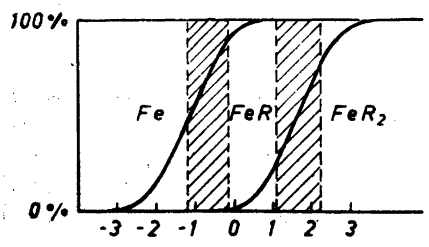


Fig. 4. Salicylamide. Distribution of Fe (III) *(in percent) over* Fe^{+3}, FeR^{+} *and* FeR_2^{-}, *as a function of* log [RH]/h.

Table 8. Salicylamide. Photometric determination of $\varkappa_1$. $[Fe]_t = [R]_t = 0.887$ mC.

h	Extinction at wave length							
	400	450	500	525	550	600	650	700
1.277	0.294	0.505	0.859	0.925	0.898	0.654	0.397	0.214
3.141	0.250	0.440	0.728	0.786	0.765	0.566	0.339	0.188
4.990	0.229	0.393	0.655	0.709	0.688	0.507	0.310	0.170
6.735	0.224	0.373	0.605	0.652	0.640	0.488	0.294	0.161
8.740	0.196	0.328	0.549	0.594	0.578	0.436	0.261	0.140
10.62	0.180	0.303	0.503	0.542	0.530	0.402	0.241	0.130
12.46	0.164	0.280	0.472	0.500	0.495	0.369	0.219	0.124
14.33	0.160	0.270	0.460	0.450	0.481	0.358	0.217	0.118

Table 9. Salicylamide. Photometric determination of $\varkappa_2$. $[Fe]_t = 0.199$ mC, $[R]_t = 3.54$ mC

h	Extinction at wave length							
	400	450	475	500	550	600	650	700
0.3430	0.117	0.220	0.290	0.336	0.318	0.212	0.110	0.060
0.2812	0.125	0.233	0.300	0.339	0.322	0.214	0.112	0.064
0.2116	0.131	0.250	0.318	0.362	0.330	0.212	0.115	0.060
0.1532	0.138	0.259	0.331	0.370	0.320	0.200	0.104	0.057
0.09493	0.162	0.310	0.380	0.410	0.338	0.199	0.094	0.046
0.04956	0.170	0.357	0.430	0.451	0.350	0.185	0.086	0.037
0.02659	0.210	0.412	0.480	0.485	0.351	0.170	0.070	0.030

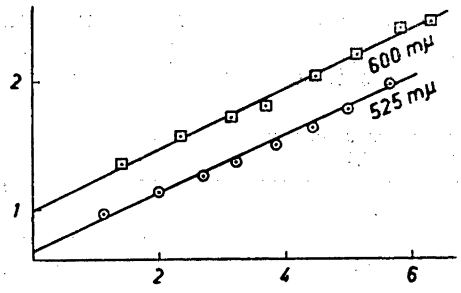


Fig. 5. Salicylamide. Photometric determination of $\varkappa_1$. c/E *vs.* $\sqrt{h/E}$. $c = [Fe]_t = [R]_t = 0.887$ mC.

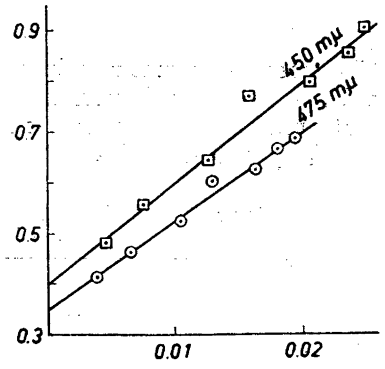


Fig. 6. Salicylamide. Photometric determination of $\varkappa_2$. $[Fe]_t/E$ *vs.* $\frac{h}{[RH]} \frac{E - \varepsilon_1 [Fe]_t}{E}$. $[Fe]_t = 0.199$ mC, $[R]_t = 3.54$ mC.

Table 10. Methyl salicylate. Potentiometric determination of $\varkappa_1$.
$[Fe]_t = 0.1032$ mC. Result: $\varkappa_1 = 0.36$ (mean value).

h	b	$\frac{[RH]}{h}$	$\frac{(b-\varkappa')h}{[RH]}$
1.627	1.3820	1.364	0.443
1.481	1.4669	1.498	0.393
1.403	1.5648	1.579	0.396
1.313	1.6770	1.687	0.387
1.182	1.8051	1.873	0.336
1.128	1.9277	1.962	0.345
1.060	2.0797	2.087	0.346
0.9845	2.2269	2.246	0.324
0.9226	2.3944	2.395	0.316

The present author [10] has determined the acidity constant of *o*-hydroxyacetophenone to be $1.5 \cdot 10^{-11}$ C, and thus their assumption must be wrong.

The solubility of *o*-hydroxyacetophenone is of the same order as that of salicylaldehyde. Thus the same restrictions have to be made here. No tendency to form the complex FeR_2 or higher complexes has been found.

The results of the potentiometric experiments are shown in Table 2 and 3. The calculated value of $\varkappa_1$ is given in Table 12. There is a remarkable difference in the results of these two experiments. However, this difference is not due to the formation of multinuclear complexes. If it were so, then the higher concentration of Fe would give a higher value of $\varkappa_1$. Instead the difference is due to the uncertainty in the results, owing to the influence of the OH-complexes on the measurements.

The photometric experiments are shown in Table 4 and Fig. 3. The calculated results are given in Table 12.

SALICYLAMIDE

The complex formation between iron(III)ion and salicylamide has been used by Hernández-Gutiérrez and Pulido-Cuchi [7] in a photometric method of determination of salicylamide. They suppose the complex to be $Fe(O \cdot C_6H_4 \cdot CONH_2)_6^{-3}$ and to be more stable than that of salicylic acid.

Table 11. Methyl salicylate. Photometric determination of $\varkappa_1$.
$[Fe]_t = 49.71$ mC, $[R]_t = 2.166$ mC.

h	Extinction at wave length							
	400	450	500	550	600	625	650	700
41.62	0.318	0.440	0.797	0.921	0.738	0.612	0.488	0.280
36.33	0.343	0.478	0.861	0.991	0.794	0.655	0.507	0.306
33.62	0.290	0.460	0.889	1.028	0.838	0.690	0.540	0.320
31.32	0.300	0.489	0.937	1.080	0.881	0.724	0.556	0.331
28.43	0.372	0.545	1.005	1.160	0.940	0.779	0.604	0.363
25.39	0.333	0.548	1.053	1.230	1.000	0.830	0.635	0.370

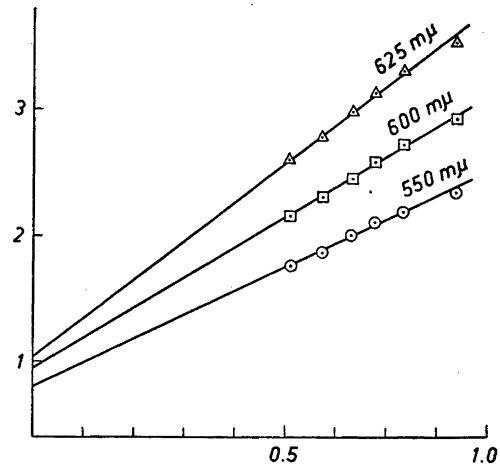


Fig. 7. Methyl salicylate. Photometric determination of $\varkappa_1$. $[R]_t/E$ *vs.* h/[Fe]. $[R]_t = 2.166$ mC, $[Fe]_t = 49.71$ mC.

Here the complex forming tendency is greater than that with either salicylaldehyde or *o*-hydroxyacetophenone, but the low solubility restricts the complex formation to FeR and FeR_2 only.

The results of the potentiometric experiments are shown in Tables 5—7, and the calculated values of $\varkappa_1$ and $\varkappa_2$ are given in Table 12.

Here the complex formation is so strong that both FeR and FeR_2 could be studied photometrically. From the potentiometrically calculated values of $\varkappa_1$ and $\varkappa_2$, Fig. 4 is constructed. From this figure, the suitable ranges of [RH]/h were chosen in which to make the photometrical experiments. These ranges are shadowed in the figure.

The results of the photometric determination of $\varkappa_1$ and ε_1 are shown in Table 8 and some of these results are used in calculating the curves of Fig. 5, equation (3) being used in the calculations. From the measurements on one of the most acidic solutions, ε_1 at other wave lengths could be calculated.

Table 12. Survey of the results of this investigation.

Substance	Potentiometry		Photometry					
	$\varkappa_1$	$\varkappa_2$	$\varkappa_1$	ε_1	$\lambda_{max.}$	$\varkappa_2$	ε_2	$\lambda_{max.}$
Salicylaldehyde	0.90 ± 0.05	$9.0 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$	1.00 ± 0.05	1.2	550	—	—	—
o-Hydroxyacetophenone	0.5 ± 0.1	—	0.50 ± 0.05	1.3	550	—	—	—
Salicylamide	13.5 ± 0.5	0.3 ± 0.1	15.5 ± 1.0	1.5	525	0.40 ± 0.05	2.9	475
Methyl salicylate	0.35 ± 0.05	—	0.40 ± 0.05	1.2	550	—	—	—

In the same manner $\varkappa_2$ and ε_2 were calculated from some of the figures of Table 9, which resulted in the curves of Fig. 6. Here equation (5) was used. The results of the photometric determinations are collected in Table 12.

METHYL SALICYLATE

The complex formation with iron(III)ion was studied by Babko [8], using a photometric method. He found the complex FeR. The dissociation constant was determined $K = \frac{[R][Fe]}{[FeR]} = 4 \cdot 10^{-8}$C. This is twice the value of the corresponding constant of phenol. The difference, according to Babko, is due to methyl salicylate being a stronger acid.

The dissociation constant of the acid methyl salicylate was determined by Babko to $1.6 \cdot 10^{-8}$. This constant has been determined by Goldschmidt and Scholz [9] to be $1 \cdot 10^{-11}$. This author [10] has found the value $6.5 \cdot 10^{-11}$ in 3 000 mC ClO_4^-. The difference to Babkos value is striking. However, if the values found by Babko are used to calculate $\varkappa_1$, defined by this author, the value 0.4 is found, which is in good agreement with the results of this author.

In this work the results of a potentiometric titration are given in Table 10 and of the photometric determinations in Table 11 and Fig. 7. The constants calculated from these results are given in Table 12.

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The Complex Formation between Iron(III) Ion and Some Phenols. IV. The Acidity Constant of the Phenolic Group

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By an ultra violet photometric method the dissociation constant of the phenolic group of some phenols has been determined. Among them, the following phenols have not been studied before: sulfosalicylic acid, *p*-amino salicylic acid, *p*-amino salicylamide and salicylamide.

In order to determine the dissociation constants of very weak acids, several methods have been used. King and Marion [1] used the catalytic effect of hydroxyl ion, Konopik and Eberl [2] made colorimetric determinations using colour indicators while photometric determinations in ultraviolet light (UV) have been used by others [3, 20]. Potentiometric or conductometric methods are not useful for the study of very weak acids.

This author has investigated the complex formation between iron(III)ion and the following phenols: sulfosalicylic acid [4], salicylic acid, *p*-amino salicylic acid (PAS)[5], salicylaldehyde, *o*-hydroxyacetophenone, salicylamide, methyl salicylate [6] and *p*-amino salicylamide (PAS-amide) [21]. In this investigation the dissociation constant of the phenolic group of these substances and of ordinary phenol has been determined. The values of these constants lie between the limits 10^{-9} and 10^{-14} C. Thus, the stronger ones of these acids could be studied by potentiometric measurements, but this could not easily be done with the weaker ones. Further, some of the substances concerned are sparingly soluble and this fact will make potentiometrical methods impractical. Hence all the substances were studied by photometric measurements in UV. Vandenbelt and coworkers [20] have demonstrated the agreement between photometric and potentiometric measurements where both could be used.

The results of previous work on the dissociation of the phenolic group of the substances studied here are collected in Table 1. Most of the substances have been studied in UV. Some recent works in this field are collected in Table 2. By comparing Table 1 and Table 2 it may be seen that in only two of the works cited have the measurements been used to determine the dissociation constant. This method has also been used by for example Flexser and coworkers [12], who studied some very weak acids and bases.

Table 1. Survey of previous investigations.

Substance	Method	Temp.	pK_a (=3−log K_a)	Ref.
Salicylic acid	Colorim.	room	13.4	2
	»	19	13.64	15
	Potentiom.	30	12.38	16
o-Hydroxyacetophenone	UV photom.	25	10.17	20
Methyl salicylate	Kinetic	25	11	17
Phenol	UV photom.	room	9.89	3
	»	25	9.87	20
	Electrom.	18	9.92	13
	Potentiom.	20	9.98	14
Salicylaldehyde	»	—	9.5	18
	(50 % dioxan) UV photom.	25	8.10	20

The authors cited in Table 1 have not used the same conditions as the present author used in his investigations on the complex formation with iron(III)-ion. The value given for methyl salicylate is very old and is said by the authors to be only approximate. For the substances studied previously by other authors, the results of the present author are given in Table 9, but no details on the measurements.

Studies of sulfosalicylic acid, PAS, PAS-amide and salicylamide in order to determine the dissociation constant of the phenolic group have not previously been performed. The results are given here in Table 9, with details in Tables 4—8.

THEORETICAL

When a phenol dissociates to phenolate there is usually a change in the UV absorption. Both the wave length of maximum extinction and maximum extinction itself become larger. In some cases there is a considerable change (about 20—50 mμ, *cf.* Fig. 4) and the dissociation is then measured at the wave length where the phenolate has its maximum extinction.

In other cases, for example salicylic acid, PAS, and PAS-amide, the wave length shift is very small (5—10 mμ, Figs. 2 and 3) but the extinction value is

Table 2. Substances previously studied in UV.

Substance	Ref.
Phenol	3, 20
Salicylic acid	8
Methyl salicylate	19
Salicylaldehyde	8, 19, 20
o-Hydroxyacetophenone	8, 20
Salicylamide	8
PAS	9, 10, 11

considerably changed. If the measurements are made at the wave length where the phenolate or phenol has its extinction maximum, the accuracy in the results will be rather small. Instead the measurements ought to be made at a wave length, where the extinction of phenolate is great and that of phenol is small. But as this is not the case near a maximum or minimum, large errors may be introduced if the wave length is only slightly changed during a series of measurements. If the apparatus is not changed the calculated value of the dissociation constant may be accurate, but the extinction value will be of little theoretical or practical interest.

The dissociation constant of a phenol is defined in the following equation

$$K_a = \frac{h\ [R]}{[RH]} \tag{1}$$

where h is the hydrogen ion concentration, [RH] and [R] are the concentration of phenol and phenolate, respectively. All concentrations are expressed in mC (= millimoles/liter). All electrical charges are omitted.

The total concentration, $[R]_t$, of the phenol is

$$[R]_t = [R] + [RH] \tag{2}$$

It is assumed that no other forms are present in measurable amounts, as for example RH_2 or RH_3. This is true for all substances studied in this work at such h-values where the phenolic group is appreciably dissociated.

The extinction, E, at a definite wave length is

$$E = \varepsilon_R\ [R] + \varepsilon_{RH}\ [RH] \tag{3}$$

where ε is extinction per mC and cm.

From equations (1)—(3) the following equation is derived

$$\frac{[R]_t}{E} = \frac{1}{\varepsilon_R} + \left[h\,\frac{E - \varepsilon_{RH}\,[R]_t}{E}\right]\frac{1}{K_a\,\varepsilon_R} \tag{4}$$

If $[R]_t/E$ is plotted against the expression within the brackets a straight line should be obtained, the intercept being $1/\varepsilon_R$ and the slope $1/K_a\,\varepsilon_R$.

If ε_{RH} is also unknown, several values may be tried. Only the right value will give a straight line (compare a previous work by this author [5]).

APPARATUS AND CHEMICALS

In the photometric measurements an Uvispek photometer with 1 cm quartz cells was used. For the determination of hydrogen ion concentration, a glass electrode was used and as a reference a silver-silver chloride electrode. In solutions with large concentrations of hydroxyl ion, a hydrogen-platinum black electrode was used. The hydrogen gas was freed from oxygen using a Meyer-Ronge [22] oven. In order to avoid evaporation of the solution to be measured, the gas was bubbled first through a 3 000 mC solution of $NaClO_4$.

The measurements were made at 25.0° ±0.1° C. Further details are described in previous works [4,5]. All experiments were made in solutions with a total concentration of 3 000 mC ClO_4^-. In this medium it is assumed that the activity factors are constant and hence that the concentrations may be used instead of activities in the equilibria.

The purity of the phenol used was controlled according to the Swedish Pharmacopeia Ed. XI. From this substance a 90 % solution was prepared and the concentration determined bromometrically according to Pharmacopeia Danica 1948. This stock solution was then diluted with water and $NaClO_4$.

Table 3. Determination of the ionic product of water, K_w.
Mean value $0.70 \cdot 10^{-8} \pm 0.05 \cdot 10^{-8}$ $(mC)^2 = 10^{-14.15 \pm 0.01}$ C^2.

$[OH]_t$	$h \cdot 10^{10}$	$K_w \cdot 10^8$
2.822	24.90	0.703
4.614	14.78	0.682
8.809	7.868	0.693
12.64	5.522	0.698
16.15	4.321	0.698
19.38	3.641	0.706
22.36	3.153	0.705
25.12	2.805	0.705
27.69	2.535	0.702
30.07	2.318	0.697
32.30	2.161	0.698

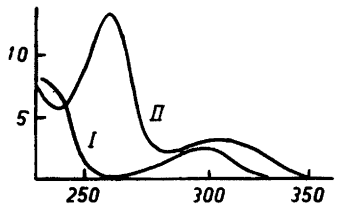

Fig. 1. Sulfosalicylic acid. ε vs. λ in mμ.
Curve I: The phenol, $^-SO_3 \cdot C_6H_3(COO^-)OH$.
Curve II: The phenolate, $^-SO_3 \cdot C_5H_3(COO^-)O^-$.

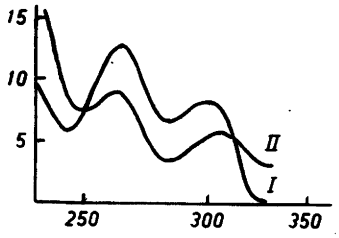

Fig. 2. PAS. ε vs. λ in mμ.
Curve I: The phenol, $NH_2 \cdot C_6H_3(COO^-)OH$.
Curve II: The phenolate, $NH_2 \cdot C_6H_3(COO^-)O^-$.

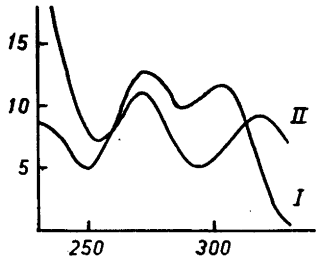

Fig. 3. PAS-amide. ε vs. λ in mμ.
Curve I: The phenol, $NH_2 \cdot C_6H_3(CONH_2)OH$.
Curve II: The phenolate, $NH_2 \cdot C_6H_3(CONH_2)O^-$.

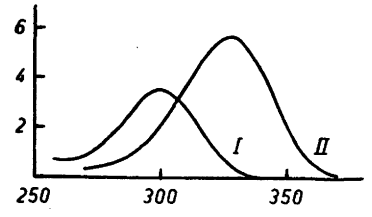

Fig. 4. Salicylamide. ε vs. λ in mμ.
Curve I: The phenol, $C_6H_4(CONH_2)OH$.
Curve II: The phenolate, $C_6H_4(CONH_2)O^-$.

Table 4. Sulfosalicylic acid. $\lambda = 260$ mμ. $[R]_t = 0.0916$ mC. In this case $\varepsilon_{RH} = 0$. Results: $\varepsilon_R = 13.3$ mC^{-1} cm^{-1}. $K_a = 1.83 \cdot 10^{-9}$ mC.

$[OH]_t$	$h \cdot 10^9$	E	$\frac{[R]_t}{E}$
0.886	9.96	0.189	0.485
1.329	6.11	0.284	0.323
1.772	4.41	0.353	0.259
2.215	3.44	0.419	0.219
2.658	2.83	0.481	0.190
3.543	2.08	0.521	0.176
4.429	1.65	0.628	0.146
5.314	1.36	0.692	0.132

Table 5. PAS. $\lambda = 325$ mμ. $[R]_t = 0.2618$ mC. Results: $\varepsilon_R = 3.1$ mC^{-1} cm^{-1}. $K_a = 1.82 \cdot 10^{-11}$ mC.

$[OH]_t$	$h \cdot 10^{11}$	E	$\frac{[R]_t}{E}$	$h \frac{E - \varepsilon_{RH}[R]_t}{E} 10^{11}$
79.0	8.86	0.191	1.37	5.84
118.5	5.91	0.243	1.08	4.33
197.5	3.54	0.320	0.818	2.82
395.2	1.77	0.440	0.595	1.51
592.8	1.18	0.525	0.499	1.03
790.4	0.886	0.561	0.467	0.783

Table 6. PAS-amide. $\lambda = 330$ mμ. $[R]_t = 0.1312$ mC. In this case $\varepsilon_{RH} = 0$. Results: $\varepsilon_R = 6.67$ mC^{-1} cm^{-1}. $K_a = 7.7 \cdot 10^{-7}$ mC.

$h \cdot 10^7$	E	$\frac{[R]_t}{E}$
18.54	0.329	0.399
6.634	0.469	0.280
3.922	0.577	0.227
2.650	0.649	0.202
1.850	0.705	0.186
1.216	0.753	0.174

Table 7. Salicylamide. $\lambda = 330$ mμ. $[R]_t = 0.1488$ mC. Results: $\varepsilon_R = 5.75$ mC^{-1} cm^{-1}. $K_a = 1.28 \cdot 10^{-6}$ mC.

$h \cdot 10^7$	E	$\frac{[R]_t}{E}$	$h \frac{E - \varepsilon_{RH}[R]_t}{E} 10^7$
14.18	0.432	0.344	12.47
10.07	0.505	0.295	9.03
6.979	0.576	0.258	6.35
5.172	0.622	0.239	4.74
4.443	0.644	0.231	4.08
3.818	0.670	0.222	3.52

Table 8. Methyl salicylate. $\lambda = 330$ mμ. $[R]_t = 0.2196$ mC.
Results: $\varepsilon_R = 5.71$ mC^{-1} cm^{-1}. $K_a = 6.5 \cdot 10^{-8}$ mC.

$h \cdot 10^8$	E	$\frac{[R]_t}{E}$	$h \frac{E - \varepsilon_{RH}[R]_t}{E} 10^8$
10.44	0.509	0.431	9.46
7.741	0.601	0.365	7.12
5.626	0.688	0.319	5.23
3.540	0.866	0.254	3.34
1.914	1.010	0.217	1.82
1.140	1.069	0.205	1.09
0.9065	1.090	0.201	0.87

PAS-amide was made according to Jensen and coworkers [23]. Its melting point was 160° C. The solutions were made up immediately before use.
The quality of the other chemicals used has been described earlier [4-6].

MEASUREMENTS

Some of the phenols studied are appreciably dissociated at rather small hydroxyl ion concentrations. In these cases the hydrogen ion concentration was determined directly. In other cases where large hydroxyl ion concentrations were needed a direct measurement of the hydrogen ion concentration was avoided. Here the h values were calculated from the stoichiometrical concentration of hydroxyl ion and the ionic product of water, the value of which was determined in the medium used. A solution of $NaClO_4$ was "titrated" with NaOH of known concentration and the hydrogen ion concentration determined with the hydrogen electrode. The results of such a titration are given in Table 3.

The extinction curves of sulfosalicylic acid, PAS, PAS-amide and salicylamide are shown in Figs. 1—4. The extinction of undissociated phenol was measured in a neutral or weakly alkaline solution. Under these conditions the carboxyl group of sulfosalicylic acid and PAS is not appreciably dissociated since the dissociation constant is 2.13 and 0.0826 mC, respectively [4, 5]. Thus, no other forms of the substances were present in measurable amounts. The extinction of the phenolate ion was calculated from the measurements in strongly alkaline solutions and equation (4).

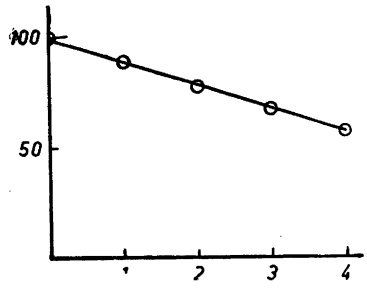

Fig. 5. Methyl salicylate. Extinction at 330 mμ (in per cent) *vs.* time (in hours).

Table 9. Survey of the results of this investigation.

Substance	K_a	pK_a (= 3−log K_a)
PAS	$(1.8 \pm 0.2) \cdot 10^{-11}$	13.74 ± 0.05
Salicylic acid	$(7.6 \pm 0.5) \cdot 10^{-11}$	13.12 ± 0.05
Sulfosalicylic acid	$(1.8 \pm 0.2) \cdot 10^{-9}$	11.74 ± 0.05
o-Hydroxyacetophenone	$(1.5 \pm 0.2) \cdot 10^{-8}$	10.82 ± 0.05
Methyl salicylate	$(6.5 \pm 0.8) \cdot 10^{-8}$	10.19 ± 0.07
Phenol	$(3.0 \pm 0.3) \cdot 10^{-7}$	9.52 ± 0.05
PAS-amide	$(7.7 \pm 0.8) \cdot 10^{-7}$	9.11 ± 0.05
Salicylamide	$(1.3 \pm 0.2) \cdot 10^{-6}$	8.89 ± 0.05
Salicylaldehyde	$(1.6 \pm 0.2) \cdot 10^{-6}$	8.80 ± 0.05

The results of the measurements in solutions of varying alkalinity are given in Tables 4 and 5 for sulfosalicylic acid and PAS, respectively. In these experiments the hydrogen ion concentration was calculated from the ionic product of water.

PAS-amide, salicylamide and methyl salicylate are stronger acids than the two foregoing ones. Here buffer solutions of sodium bicarbonate and sodium hydroxide were used to fix the hydrogen ion concentration. The measurements in these cases were made with the glass electrode or the hydrogen electrode. The results are given in Tables 6—8.

These three substances are not quite stable in alkaline solutions but may be hydrolyzed to the corresponding carbonic acid. If the change in extinction is due to hydrolysis the change is supposed to be slow and should not reverse when the solution is acidified again. The amides were studied in this respect but no change could be found within three hours. When acidified again the calculated extinction value was obtained.

The change in extinction may be due to a dissociation of the amide group. When benzamide was studied no change in extinction could be found even in very strongly alkaline solution. Thus this reaction is improbable.

Methyl salicylate is hydrolyzed more easily under the conditions used here. In order to estimate the speed of this reaction the extinction of one solution in 100 mC NaOH was measured during four hours. In Fig. 5, the decreasing values (in per cent) obtained are shown. For the determination of the dissociation constant more weakly alkaline solutions were used and the extinction was measured within five minutes. During this time the hydrolysis is surely not greater than 1 % and thus does not appreciably influence the results.

All the dissociation constants determined in this work are collected in Table 9.

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Half-Life and Radiations of the Long-Lived Isotope of Niobium (^{94}Nb)

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In order to investigate the half-life and radiation energies of ^{94}Nb, spectroscopically pure metallic niobium was irradiated for 23 days with thermal neutrons in the JEEP pile. The short-lived 6.6 minutes ^{94m}Nb beta activity was confirmed. The bulk of the slow decaying activity is due to ^{182}Ta beta and gamma radiations. Many ways were tried to free efficiently the niobium metal from the tantalum impurity: anion exchange column, coprecipitation of Ta as potassium fluotantalate and Ta/Nb solvent extraction using di-*iso*propyl ketone. Only the last of these proved successful. The residual activity of the purified Nb_2O_5 is very low. From an Al-absorption curve the beta energy can be estimated to be between 0.5 and 0.6 MeV. The gamma spectrum, investigated with a scintillation spectrometer, points to three energies, namely 0.73 MeV, 0.90 MeV and 1.63 MeV. The counting rate of the beta activity, corrected for counting efficiency, indicates a half-life for the ^{94}Nb of $1.77 \cdot 10^4$ years ($\pm$ 25 %).

The formation of a 6.6 minutes activity in neutron irradiated niobium has been reported by several authors [1]. This activity has been ascribed to an isomeric state, ^{94m}Nb, which, except for 0.1% beta decay leading to ^{94m}Mo, decays by gamma emission to a more stable ^{94}Nb. The properties of the latter long-lived nuclide had, until recently, not been observed directly but only inferred from other experimental data. Thus some authors [2] assigned to it a beta decay and a half-life much greater than 100 years, while others [3] indicated a lower limit of the half-life of $5 \cdot 10^4$ years. The aim of the present work, of which a preliminary report has already been given [4], was to investigate the beta emission of the long-lived ^{94}Nb and assign to it a better approximated half-life. The recent work by Douglas, Mewherter and Schuman [5] on the long-lived niobium activity was not known to us, our results obtained independently and with different methods confirm the findings already reported by these authors.

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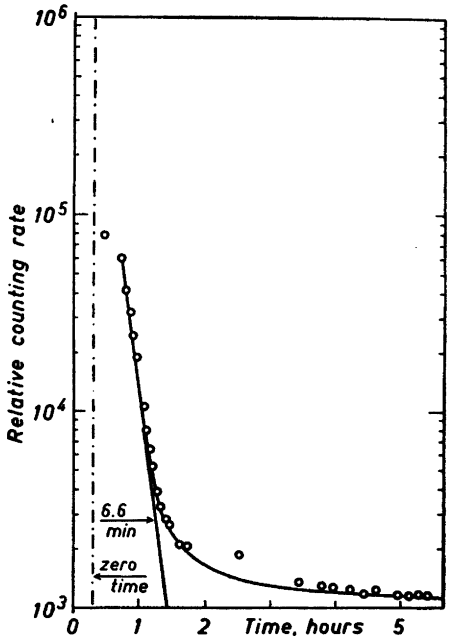

Fig. 1. Decay curve of the 6.6 minutes half-life 94m niobium.

IRRADIATIONS AND CHEMICAL SEPARATION METHODS

Many pure niobium salts have been irradiated in the JEEP pile and in particular: (a) niobium pentoxide obtained from Kahlbaum's K_2NbF_7, (b) niobium pentoxide obtained by hydrolysing niobium pentachloride of Schuchardt, and (c) spectroscopically pure niobium metal of Johnson &

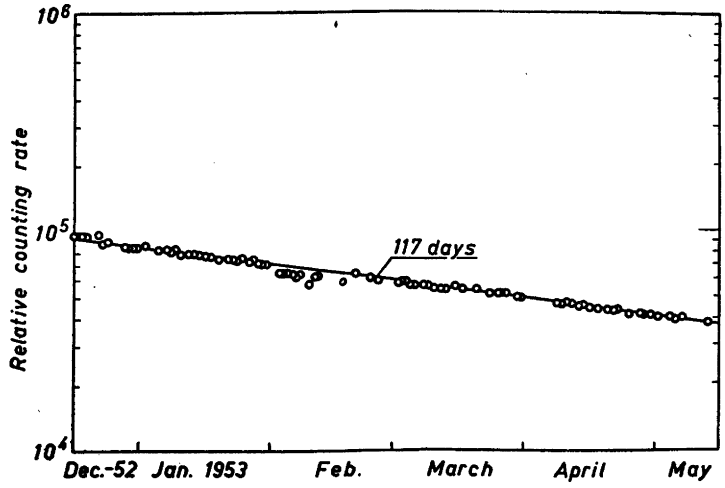

Fig. 2. Decay curve of the irradiated specpure niobium without any previous chemical processing. The 117-day half-life is due to ^{182}Ta.

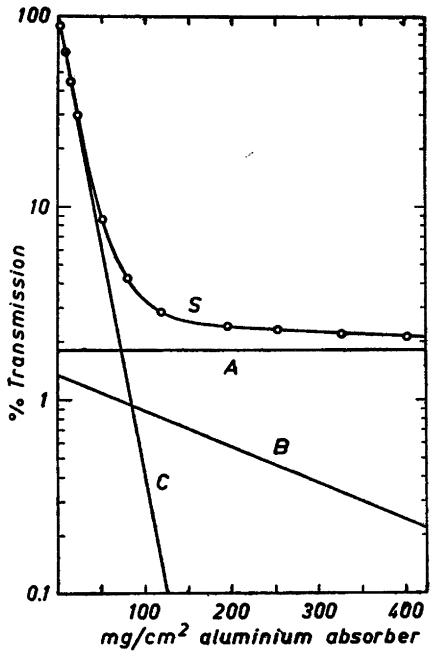

Fig. 3. Aluminium absorption curve of irradiated specpure niobium after (insufficient) ion-exchange purification. S experimental absorption curve, A hard gamma background, B soft gamma, C 0.53 MeV beta activity. (Counting tube Amperex 100 CB, sample-to-window distance 9 mm.)

Matthey. Two irradiations were carried out, a short one of half an hour, in order to follow the decay of the short-lived ^{94m}Nb, and a long one lasting 23 days, in order to accumulate a fairly good amount of the long-lived nuclide.

From the decay curve of a metal sample irradiated for a short time the half-life of the ^{94m}Nb was confirmed, the value being 6.6 minutes (Fig. 1). In the absence of any chemical processing the decay curve, followed for 5 months on a metal sample irradiated for 23 days, was a straight line giving a half-life of 117 days (Fig. 2). This half-life belongs to the 182 tantalum activity. It is easy to draw the conclusion that, owing to the large cross section of 21 barns of natural 181 tantalum (as compared to the cross section of 1.1 barns for the natural occurring niobium) and the respective half-lives of the produced nuclides, this activity should be present, even for a very small tantalum impurity in the spectroscopically pure niobium metal. By a simple activation analysis the tantalum impurity in this sample was found to be of the order of 0.2 %.

It thus appeared necessary to purify the irradiated niobium and this was tried in many ways:

(A) Ion-exchange column. The dissolved niobium (4 g) was treated in an anion-exchange column using a hydrofluoric-hydrochloric solution as elutriant. The process is rather time-consuming. Most of the niobium is collected in the first fractions whereas tantalum is eluted later.

The solid matter contents of one of the first fractions evaporated to dryness still showed an activity that had to be assigned to the ^{182}Ta after an aluminium absorption curve was taken. It has been reported [6] that ^{182}Ta is perhaps the

most prolific emitter of electron lines of all the known radioactive isotopes. A lead absorption curve taken of the activity of this column fraction gave an absorption coefficient $\mu_x = 0.7$ cm^{-1} equivalent to an energy of 1.12 MeV. This is the energy[7] of the most intense λ-radiation emitted by ^{182}Ta. A further purification thus became necessary. One of the first fractions gathered from the ion exchange column was dried, redissolved, a large amount of hydrofluoric solution of pure tantalic acid (H_3TaO_4) added and the tantalum precipitated as potassium fluotantalate. This treatment was repeated twice, a large amount of active tantalum being eliminated in the precipitate. The niobium was precipitated from the filtrate, purified by complexing with oxalic acid [8], and finally precipitated as Nb_2O_5, dried on a Pt disc, and ignited in a crucible for 5 hours. 5 ½ months after the end of the irradiation, a sample thus treated still showed a considerable specific activity. The activity measured was 102 c/m per mg Nb_2O_5 counted on shelf 2 of a standard Geiger-counter sample holder (9 mm sample-to-window distance) using an Amperex tube model 100 CB.

An Al absorption curve, S, was plotted (Fig. 3). After subtracting the hard gamma background, A, (1.12 MeV from ^{182}Ta) two components were left: a component B of 160 mg/cm^2 half-thickness and a beta component C of 14 mg/cm^2 half-thickness. The latter is due to the 0.53 MeV beta activity of ^{182}Ta and the former to the softer λ-radiations from ^{182}Ta.

No new activity from ^{94}Nb was apparent. A second Al absorption curve of the same sample, taken about 4 months later (9 months and 19 days after removal from the reactor) when the specific activity, with the same counter geometry, had reduced to about 49 c/m per mg Nb_2O_5, gave exactly the same results.

(B) A second attempt to free 850 mg of irradiated niobium from ^{182}Ta activity by ten successive additions of decreasing amounts of dissolved natural tantalum, each followed by a tantalum precipitation as K_2TaF_7 yielded no better results. The end product was a niobium pentoxide sample with specific activity, at same geometry, of 87 c/m per mg Nb_2O_5, measured 12 months and 18 days after removal from the reactor.

(C) The decisive results were obtained by the procedure due to Stevenson and Hicks [9], of separating tantalum from niobium by extraction into certain polar organic solvents. The solvent used was di-*iso*propyl ketone. The aqueous solution used was 0.4 *M* HF, 3 *M* HCl, in which irradiated niobium and inactive tantalum, transformed into the respective fluorides, were dissolved. 4 successive extractions were carried out in this way. After the 3rd extraction it seemed difficult to free the precipitate from some chemical impurity introduced during the extractions. The precipitate was therefore purified by complexing the niobium with oxalic acid and treating the solution with concentrated nitric acid and $KBrO_3$. The same purification was performed after the 4th extraction.

14 months after the end of the irradiation the final purified and ignited Nb_2O_5 showed a relative specific activity of 14.3 c/m per mg Nb_2O_5 counted on shelf 1 as compared to 13 000 c/m per mg Nb_2O_5 for the untreated sample. In order to compare these figures with those previously given for the separation methods A and B, it should be noted that 14.3 c/m on shelf 1 is equivalent to 9.5 c/m on shelf 2. The decay of this residual activity was further followed

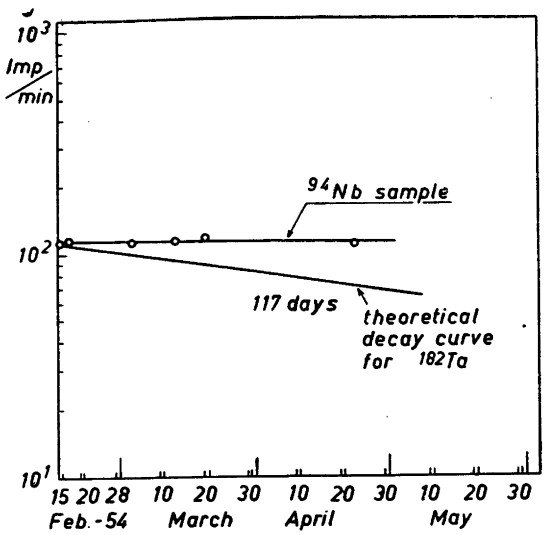

Fig. 4. Decay curve of irradiated niobium purified by solvent extraction (Method C). Constant activity indicates absence of ^{182}Ta.

for more than 2 months but no decrease in the activity (indicating ^{182}Ta) was detectable (Fig. 4).

The radiation energies and half-life of this long-lived activity were then determined in the following way.

ENERGY OF RADIATIONS

An aluminium absorption curve of the residual activity in a Nb_2O_5 sample resulting from the separation method (C) is shown in Fig. 5. The curve indicates a beta component with a half-thickness in Al of about 15.5 mg/cm^2 corresponding to a beta energy of 0.5—0.6 MeV (*cf.*[10]). No very exact determination of the beta energy was attempted because of the weakness of the sample which made difficult the correction for the contribution of the hard component measuring only a few counts per minute.

The gamma activity was first roughly checked by an ordinary gamma scintillation counter. A lead absorption curve indicated a gamma energy of about 0.9 MeV. The detailed investigation of the gamma spectrum was done by Dr. E. Germagnoli first at Kjeller and later at the laboratories of C. I. S. E., Milan, Italy. The final results were obtained using a 1'' NaI crystal, a phototube Dumont, a model 100 (Los Alamos type) linear amplifier and the 99 channel built by Gatti [11].

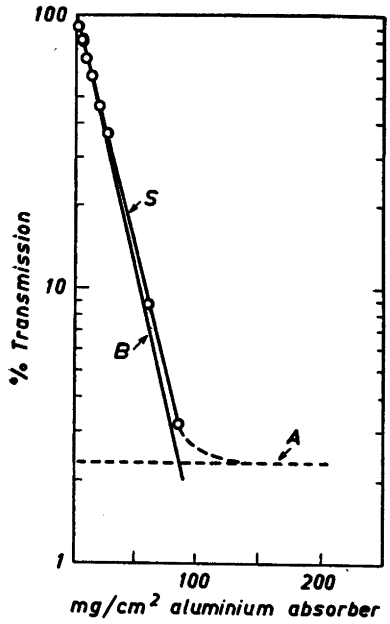

Fig. 5. Aluminium absorption of the sample purified by solvent extraction. S experimental absorption curve, A ~ 2.5 % gamma contribution, B beta contribution probably due to ^{94}Nb. (Counting tube Amperex 100 NB, sample-to-window distance 14.5 mm.)

The scintillation spectrogram obtained is shown in Fig. 6 a and the energy calibration by means of the radiations from ^{60}Co and ^{137}Cs in Fig. 6 b. The following gamma energies are found:

$$E_1 = 0.726 \text{ MeV}$$
$$E_2 = 0.903 \text{ MeV}$$
$$E_3 = 1.65 \text{ MeV (measured)}$$
$$1.63 \text{ MeV (as } E_1 + E_2)$$

These results are in good agreement with the values obtained by Douglas, Mewherter and Schuman [5], namely: $E_\beta = 0.50 \pm 0.05$ MeV, $E_1 = 0.70 \pm 0.01$ MeV, $E_2 = 0.87 \pm 0.01$ MeV and $E_3 = 1.57 \pm 0.02$ MeV.

SPECIFIC ACTIVITY AND HALF-LIFE

The knowledge of the absolute disintegration rate of the sample containing long-lived ^{94}Nb is needed for its half-life determination.

The absolute beta counting, using an end-window Geiger tube, was carried out according to the procedures described by Zumwalt and Burtt and computing the absolute number of disintegrations per minute (d/m) according to the well known formula [12, 13].

$$\text{d/m} = \frac{(\text{c/m})_{\text{extrap.}} \cdot 100}{G \cdot f_A \cdot f_B \cdot f_H \cdot f_{ssa}}$$

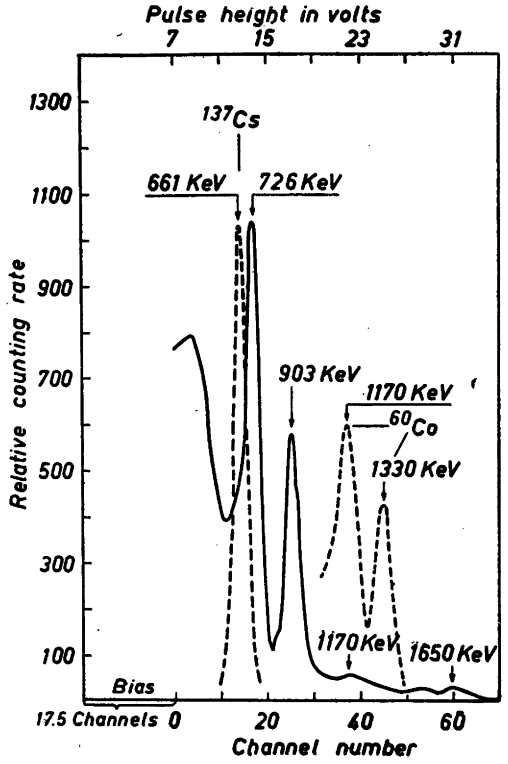

Fig. 6 a. Scintillation spectrogram of gamma radiation from irradiated niobium purified by solvent extraction (Method C). The dotted lines give the peaks of ^{137}Cs and ^{60}Co which were used as references.

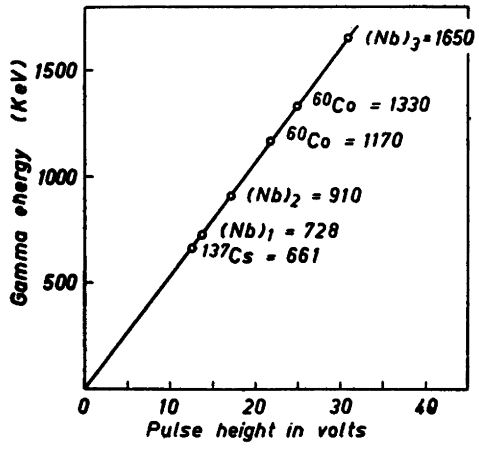

Fig. 6 b. Calibration curve used for the scintillation spectrogram giving the gamma energy as a function of pulse height.

where:

$(c/m)_{extrap.}$ = observed counts per minute corrected for coincidence and background and extrapolated from an Al absorption curve to zero absorber.

G = geometry of the counting set up.

f_A = factor for the effect of air in scattering beta particles into the counter.

f_B = factor for the increase in counting rate due to backscattering by the material supporting the source.

f_H = housing effect (support and walls of source housing).

f_{ssa} = correction factor for the self-scattering and self-absorption due to thickness of sample.

The 12.1 mg sample counted in Milan with an Amperex 100 CB end-window tube gave an observed counting rate of 113.36 c/m at 9 mm sample-to-window distance. No gammas were detected during this measurement owing to the low sample activity. Introducing the theoretical correction factors [12, 13] the value of 875 d/m for the absolute disintegration rate of the sample was first obtained and from this a half-life of $2.7 \cdot 10^4$ years was computed and reported in our preliminary communication [4]. Intercomparison of the same sample and countings performed at Kjeller lead to the qualitative detection of the gamma radiation and also revealed that a too high counting efficiency had been assumed for the counting performed in Milan. After a careful check we had to assign an overall counting efficiency of only 8.26 % for this measurement. This gives for the absolute disintegration rate of the 12.1 mg sample:

$$\mathrm{d/m} = \frac{113.36 \cdot 100}{8.26} - 2.5\ \% = 1\,337$$

where the 2.5 % subtracted is due to the gamma activity.

The specific activity, S, in curies per g of the target niobium metal, freed from any other activity due to impurities is given by:

$$S = \frac{(\mathrm{d/m}) \cdot 10^3}{60 \cdot a \cdot 3.7 \cdot 10^{10}}$$

where a (= mg Nb of measured sample) is obtained subtracting 2 % (due to inactive Ta_2O_5 left from the ketone extraction) from the sample weight and multiplying by the factor 0.7 (as Nb_2O_5 contains 70 % Nb). This gives $a = 8.29$, thus

$$S = \frac{1.337 \cdot 10^6}{60 \cdot 8.29 \cdot 3.7 \cdot 10^{10}} = 7.25 \cdot 10^{-8}\ \text{curies/gram}$$

For active nuclides whose half-life, as in this case, is long compared to the irradiation time, the simplified formula for calculating the half-life is:

$$T_d = \frac{1.136\ \sigma\ F\ t}{A\ S}$$

where

T_d = half-life in days of the nuclide formed
t = irradiation time in days
F = neutron flux in neutrons per cm^2 per second $\times 10^{-11}$
σ = activation cross section in barns = 1.1 [14]
S = specific activity in curies/gram of the target element on removal from the pile
A = atomic weight of the element = 93

The average flux during the 23 days irradiation was $1.5 \cdot 10^{11}$ n cm^{-2}sec^{-1} thus:

$$T_d = \frac{1.136 \cdot 1.1 \cdot 1.5 \cdot 23}{93 \cdot 7.25 \cdot 10^{-8}} = 6.4 \cdot 10^6 \text{ days}$$

corresponding to a half-life of $1.77 \cdot 10^4$ years.

Taking into account the approximate values used it is safe to assume that the half-life of ^{94}Nb is not determined with an accuracy greater than $\pm$ 25 %. The value found is in good agreement with the value of $(2.2 \pm 0.5) \cdot 10^4$ years proposed by Douglas, Mewherter and Schuman [5].

EXPERIMENTAL

(A) The ion-exchange column used with hydrofluoric elutriant solution was made of plastic tubing of 3 mm diameter, 7.1 mm^2 cross section and 450 mm length. The ion-exchange resin was Dowex No. 1. A small finely perforated platinum disc closed the bottom of the column and held the resin. All the tubing was made of polyvinyl plastic and the containers of polythene. In front of the column, driven by a small electric motor so that it could move along the whole length of the column in about 15 minutes time, was a Geiger counter connected with a scaler, rate-meter and a Brown recorder. This made possible the automatic recording of the activity as a function of the position in the column. The ion-exchange resin was converted to the chloride form and the niobium contaminated with tantalum activity was added in the form of niobium chloride solution. Up to 150 mg of $NbCl_5$ were added at a time. This ion-exchange process has already been described elsewhere [15]. For the solution used (9 M HCl, 0.5 M HF) the elution constants were:

$$E_{Nb} = 70 \qquad E_{Ta} = 5$$

so that the ratio was $E_{Nb}/E_{Ta} = 14$

in which $E = d \times A/V$, where d = length in cm covered by activity maximum after V ml of elutriant have passed through a column the cross section of which is A cm^2. A lead screen provided with a small central slit, was mounted on the Geiger counter window. The motor was provided with a reverse gear and a speed reductor. The fractions were collected in polythene containers in which they were dried under a heat-lamp, treated many times with HCl in order to get rid of the hydrofluoric acid, and then mounted on standard watch glasses and counted. The elution speed was kept at about 1 ml in 20 minutes. In a second ion-exchange column of 7 mm diameter and 38.5 mm^2 cross section the elution speed was kept at 8 ml for 30 minutes. The automatically recorded elution curves are collected in Fig. 7.

The precipitation of 182 tantalum with pure natural tantalum as a carrier was carried out collecting the fractions containing niobium from the ion-exchange procedure. These fractions were dried and then dissolved in 1/1 solution of conc. HNO_3 and H_2F_2. To this solution a hydrofluoric solution of pure H_2TaO_4 was added and the tantalum then precipitated as potassium fluotantalate by addition of KHF_2. The precipitate was centrifuged and the solution treated a second time in the same way.

Fig. 7. Automatically recorded activities of the ion-exchange column containing 182 tantalum. The number near each curve indicates the number of hours elapsed from the beginning of the elution, till the moment the column was recorded.

The first precipitate was very active as measured with a GM-survey meter, the second one much less so. After the tantalum precipitation the acidic niobium solution was neutralized with ammonium hydroxide, in order to precipitate the niobic acid $HNbO_3$. This was centrifuged and thoroughly washed with ammonium hydroxide and water. The precipitate was then dissolved in 2 ml of saturated oxalic acid solution to which 10 drops of concentrated nitric acid had been added. The complexing action of oxalic acid on niobium leads to a complex of the type $M_3(NbO(C_2O_4)_3)$ which is often hydrated. In this case the soluble acid complex $H_3(NbO(C_2O_4)_3)$ is found which is hydrated with a number of water molecules ranging from 1 to 4. The complex is stable in the presence of an excess of oxalic ions, but it is easily hydrolyzed and Nb_2O_5 is precipitated when these are destroyed. In order to obtain this result 1 g of $KBrO_3$ was added and the precipitated niobic acid was centrifuged, washed with 6 *N* HNO_3, with 6 *N* NH_4OH and with distilled water. A fraction of this precipitate was slurried over a platinum disc, dried with a heat-lamp and then calcinated. The thin film of Nb_2O_5 left was heated for 3 hours at a light red heat in a furnace to get rid of all possible volatile impurities.

(C) The only successful Ta/Nb separation was carried out by extraction with di-*iso*-propyl ketone using a technical grade reagent from the British Drug Houses Ltd. Stevenson and Hicks [9] reported that tantalum goes into the organic layer and that, starting with equal amounts of Ta and Nb, the precipitate from the aqueous layer after one extraction contains 98 % Nb and 2 % Ta as the pentoxides.

In our first extraction 200 mg irradiated niobium metal were fluorinated with conc. HF and 7 *M* HNO_3, dried, and 90 mg Ta added in the form of TaF_5 (made from Kahlbaum's K_2TaF_7). The fluorides were dissolved in 50 ml 0.4 *M* HF, 3 *M* HCl solution and extracted twice with 40 ml di-*iso*propyl ketone. The organic layer was discarded, and the aqueous layer added to 4 ml saturated H_3BO_3 solution in a centrifuge cone, in order to complex any fluoride present. Ammonium hydroxide was added to precipitate the hydrated oxide

of niobium. This was washed with diluted NH_4NO_3 solution, centrifuged and dissolved again in 25 ml 0.4 *M* HF, 3 *M* HCl solution in which 45 mg Ta were also dissolved. The second extraction was carried out with 2 times 25 ml of di-*iso*propyl ketone, and the precipitation from the aqueous layer repeated. The 3rd extraction was performed adding 30 mg Ta, dissolving in 15 ml acid solution, and extracting twice with 15 ml ketone.

The precipitate from the 3rd extraction was purified by complexing with oxalic acid, as mentioned earlier, (p. 60), and ignited. From this Nb_2O_5 a sample containing 15 mg Nb was fluorinated again, 15 mg Ta in the form of TaF_5 added, and both dissolved in 7.5 ml 0.4 *M* HF, 3 *M* HCl solution. For the 4th extraction the solution was treated twice with 7.5 ml ketone and the whole procedure repeated, including purification by complexing with oxalic acid. The sample was carefully ignited on a platinum disc before being transferred to an aluminium disc for beta counting. Using the data of Stevenson and Hicks [9] we calculate the composition of the final sample to be 97.9 % Nb and 2.1 % Ta. This is taken into account when calculating the specific activity of the niobium from the activity of the sample.

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Magnetic Measurements on Crystallized Fe-Transferrin Isolated from the Blood Plasma of Swine

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The paramagnetic susceptibility of the iron in Fe-transferrin is determined to be 15 700 ($\pm$ 500) · 10^{-6} cgs emu at 20° C, which corresponds to an effective magnetic moment of 6.08 ($\pm$ 0.10) Bohr magnetons. The iron is thus trivalent and bound with essentially ionic bonds. Some magnetic interaction between the two iron atoms in each protein molecule is possible. After reduction and decolorisation with hydrosulfite the iron is present as free or bound divalent ions with an effective moment of 5.1 Bohr magnetons. The diamagnetic gram susceptibility of transferrin was determined to be -0.586 ($\pm$ 0.007) · 10^{-6} cgs emu.

The biochemical and physico-chemical properties of the serum protein, transferrin (siderophilin), and its role as an iron transporting factor in blood have recently been reviewed [1]. The molecular weight of transferrin is about 90 000 [2,3], and it is capable of reversible combination [2,4,5] with two atoms of iron per molecule of protein [2,3,6]. The compound between iron and transferrin, Fe-transferrin, is optically characterized by a relatively broad absorption band with its maximum at 470 mμ. The formation of Fe-transferrin when di- or trivalent ionic iron is added to a solution of transferrin can thus be followed colorimetrically. Such experiments disclose the interesting fact that in plasma under aerobic conditions the Fe-transferrin is formed more rapidly with ferrous than with ferric ions [5], while in solutions of purified transferrin the ferric ions react faster than the ferrous ions do [4,6,7]. Colorimetrically judged the formed compound is identical in all these cases. The effect might be due to different rates of autoxidation of ferrous iron [6].

Since most chemical evidence points in the direction that the iron of Fe-transferrin is trivalent, this is commonly believed to be the case. No direct proof of this hypothesis has yet been obtained, and the mode of binding of iron in Fe-transferrin is still obscure. Michaelis, however, made magnetic measurements on Fe-transferrin, but his results are only published as a personal communication to Koechlin [6] and no experimental data are given. Koechlin simply states that Michaelis had found the iron to be trivalent in the red complex, and that he also had found "that hydrosulfite reduced the ferric iron and dis-

charged the red color in the complex at reactions where dissociation of the complex would not normally have occurred".

For further discussion of the role of transferrin in the iron transporting mechanism of the blood it is important to know as much as possible about the state of iron in the compound, Fe-transferrin. As the above statements are only qualitative in nature, we have reinvestigated the magnetic properties of Fe-transferrin in a highly purified form.

MATERIAL AND METHODS

Iron-saturated Fe-transferrin was prepared from swine plasma according to Laurell [8] and recrystallized four to six times. During the steps of the preparation, which were performed at a pH below 6, a small amount of ferrous ammonium sulfate was added to prevent dissociation of the Fe-transferrin compound. The excess of iron was eliminated during the later preparative steps. Since it was difficult to remove all of the non-specifically bound iron in this way, one sample (No. 2) was run through a column containing the ion exchange resin, IRA 120, pretreated with $M/15$ phosphate buffer of pH 7.8. In this way the iron content was brought to 0.125 %. An iron content of 0.124 % is to be expected from a molecular weight of 90 000 and two atoms of iron per molecule. The other samples contained 0.127 to 0.134 % iron (See Table 1). The recrystallizations were repeated until no more hemi-muco-β-globulin could be spectrophotometrically detected [8].

To get iron-free transferrin for the determination of its diamagnetism, one sample of Fe-transferrin was dialysed at pH 3.5 in the presence of cysteine and *o*-phenanthroline. After twenty-four hours all the iron was in the form of the ferrous *o*-phenanthroline complex, which was dialysed away against glass-distilled water.

All samples were analysed for dry weight (at 110° C) and for iron. For the iron determinations two colorimetric methods were used, with sulfo-salicylic acid or with *o*-phenanthroline as complexing agents. They gave closely agreeing values in all cases.

The extinction values were determined in a Beckman spectrophotometer, Model DU. The magnetic measurements were carried out with the apparatus constructed by Theorell and Ehrenberg and a solution of nickel chloride was used as calibration agent [9]. All measurements were made in the temperature range 20 to 22° C.

RESULTS

Four samples of Fe-transferrin from different preparations were investigated for the paramagnetism of the iron by measurements on their salt-free solutions. The results are given in Table 1 together with the characteristics of the samples. The extinction at 470 mμ is proportional to the amount of specifically bound iron. Sample *2* contains practically all of its iron as Fe-transferrin compound. Samples *3* and *4* are saturated with specifically bound iron and contain above that level respectively 2.9 and 9.0 % of iron non-specifically bound to the protein. Sample *1* may be impure or partly denatured since, according to the extinction value, 9 % of its specific sites are unoccupied by iron in spite of an iron content slightly above the theoretical saturation level.

The susceptibility values are calculated per gram atom of iron and reduced to 293° K with the assumption that Curie's law is valid. The spreading of the separate determinations on every sample gives a standard deviation of the mean of about $\pm 150 \cdot 10^{-6}$ cgs emu. Another error of $\pm 350 \cdot 10^{-6}$ cgs emu is due to uncertainties in the diamagnetic correction and has the same sign in all determinations. As is seen, the different and small amounts of non-specifically bound iron do not influence the average susceptibility to any detect-

ible extent. The non-specifically bound iron has thus much the same susceptibility as the specifically bound. As a mean value of the susceptibility of the iron in Fe-transferrin we accept 15 700 ($\pm$ 500) $\cdot$ 10^{-6} cgs emu at 293° K. This corresponds to an effective magnetic moment, μ_{eff} = 6.08 ($\pm$ 0.10) Bohr magnetons. Preliminary measurements on three samples, containing slightly less iron than those described in the table, also indicated a μ_{eff} of about 6.1. No foreign heavy metal ions that could contribute to the paramagnetism of the solution could be detected by common chemical methods.

One part of sample No. *4* was diluted with phosphate buffer of pH 6.5 to final concentrations of 3.19 % protein and *M*/30 phosphate. When hydrosulfite, 5 mg per ml solution, was added, the solution started to decolorize rather slowly. The extinction values in the wavelength region, 420—600 mμ, decreased in proportion. The process could not be followed to the end as turbidity occurred. When the extinction at 470 mμ had diminished to 50 %, the solution, which was still clear, was measured in the magnet. The result showed that the decolorized iron had a susceptibility of 11 000 $\cdot$ 10^{-6} cgs emu corresponding to μ_{eff} = 5.1 Bohr magnetons.

Table 1.

Sample No.	Protein conc. mg/ml	Fe conc. γ/ml	Fe/protein ‰	$\beta_{470m\mu} \times 10^{-7}$ cm²/g AtFe	Number of magnetic determ.	$\chi_{Fe,\ 293°\ K}$ 10^{-6} cgs emu
1	27.3	34.7	1.27	0.486	4	15 900
2	22.7	28.4	1.25	0.556	4	15 500
3	44.5	56.4	1.27	0.540	4	15 800
4	63.8	85.2	1.34	0.506	3	15 600

The diamagnetism of the protein was determined by a series of five measurements on an "iron free" sample with 21.4 mg protein per ml and 2.0 γ iron per ml. The mean difference reading on the micrometer could be used immediately for diamagnetic correction of the readings in the experiments already described, if the iron contents were properly reduced because of the small but not negligible iron content of the "iron free" sample. To evaluate the true diamagnetism of the protein we have corrected for the paramagnetic contribution of this iron. For the partial specific volume of transferrin we take the figure 0.725, determined by Oncley, Scatchard and Brown [10] on a 70 % pure material. If we assume that Wiedemann's additivity law is applicable and that the gram susceptibility of water is equal to —0.7200 $\cdot$ 10^{-6} cgs emu we find the gram susceptibility of transferrin to be —0.586 ($\pm$ 0.007) $\cdot$ 10^{-6} cgs emu at 293° K. A small correction has been made, due to the physically dissolved oxygen, but it does not amount to more than one unit in the third decimal.

DISCUSSION

As already mentioned the transferrin molecule is capable of combining with two atoms of iron. There is some doubt whether this reaction takes place in one step or two, or, whether the active sites of the molecule are close enough

to each other to bring about an interaction between them [11]. Different possible structures have to be proposed and their theoretical magnetic moment has to be compared with the value $\mu_{eff} = 6.08$ ($\pm$ 0.10) B. m. (Bohr magnetons) obtained in the present investigation.

We first assume that *no interaction* exists between the two iron atoms bound to the same protein molecule. In this case the possibility of divalent iron can be ruled out at once, as it would exhibit a maximal effective moment of 4.90 B. m. or slightly higher, due to incomplete quenching of the orbital moment. All experimental values of μ_{eff} of ionic ferrous iron in magnetically dilute solids or solutions lie between 5.1 and 5.6 B. m., which is definitely lower than the 6.08 B. m. of Fe-transferrin. Ferric iron, however, would give $\mu_{eff} = 5.92$ B. m., if it is bound with essentially ionic bonds. Experimental values are often close to that figure, but usually lower, probably due to deviations from the Curie law. Higher values seem to be rare, if any. Because of the small difference between 6.08 ($\pm$ 0.10) B. m. and 5.92 B. m. a structure with ionic trivalent iron is rather possible in the case of Fe-transferrin.

The other extreme possibility is that *complete magnetic coupling* occurs between the two iron atoms in Fe-transferrin. This means that they can be treated as an entity and, if the electronic spins only are magnetically active, the effective magnetic moment per iron atom can be calculated according to the formula $\mu_{eff} = \sqrt{\frac{1}{2} n(n+2)}$ B. m., where n is the total number of odd electrons in the entity. Two trivalent iron atoms with five odd electrons each would thus give $\mu_{eff} = \sqrt{60} = 7.75$ B. m. The large difference between this figure and the experimental one makes the proposed structure improbable. Two trivalent iron atoms, one with five and one with three odd electrons, or two divalent iron atoms with four odd electrons each would both give $\mu_{eff} = 6.33$ B. m. Since the orbital contribution has been overlooked, the figure 6.33 is probably somewhat too low but anyhow close enough to the experimental value to require discussion. The strong magnetic coupling assumed here calls for a rather compact and stable structure, especially in the case of trivalent iron, where a kind of resonance between the iron atoms must be postulated. Such a stable structure is not easy to reconcile with the fact that the combination between iron and transferrin is an easily reversible reaction. From a chemical point of view the case of divalent iron is further improbable. On these grounds we believe that strong interaction does not occur between the iron atoms of Fe-transferrin.

The best explanation of the experimental value $\mu_{eff} = 6.08$ ($\pm$ 0.10) B. m. seems to be that the iron in Fe-transferrin is trivalent and bound with essentially ionic bonds. The fact that the observed value is higher than the expected one of 5.92 B. m. might be interpreted as due to a weak interaction between the two iron atoms in each protein molecule, which in such a case may not be situated too far from each other. To illuminate the proposed interaction further magnetic measurements over a temperature range and detailed studies of the coupling reaction between ionic iron and transferrin are highly desirable.

The value $\mu_{eff} = 5.1$ B. m. obtained for the iron after decolorisation is just what would be expected for divalent ionic iron. We can thus state that if transferrin forms a compound with ferrous iron, it is either uncolored and ionic or it is unstable in the presence of phosphate ions at pH 6.5.

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Studies on Arsenic Trichloride as a Solvent

II. On the Constitution of Solutions in Arsenic Trichloride

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The importance of ionic transfer processes in acid-base reactions in $AsCl_3$ is discussed, and the way in which they differ from Lewis' acid-base concept is pointed out. A program for experimental studies to explore the constitution of solutions in arsenic trichloride is suggested. It is partly based on a discussion of the possible similarities with aqueous solutions.

ACID-BASE CONCEPT

In connection with studies on non-aqueous solvents three main acid-base concepts have been advanced: the solvent system concept, the protonic concept and the electronic theory. They are excellently reviewed by Audrieth and Kleinberg [1] in the book "Non-aqueous solvents". In a recent paper [2] a development has been made of the solvent system concept, focussing the interest on the ionic transfer processes possible in different solvents. This represents at the same time an extension of the protonic concept to non-protonic solvents. Flood's [3] treatment of oxide melts is also included.

The new concept defines acids and bases in $AsCl_3$ in the following way: acid + Cl^- = base (*cf.* acid = base + H^+) *or* base + solvent = acid + Cl^- (*cf.* acid + solvent = base + H^+ in some cases [5]).

$AsCl_3$ is an ampholytic solvent and can react as an acid as well as a base: $AsCl_3 + Cl^- = AsCl_4^-$ and $AsCl_3 = AsCl_2^+ + Cl^-$. It has therefore a characteristic auto-ionization: $2\ AsCl_3 = AsCl_2^+ + AsCl_4^-$, which is the basis for the solvent system definition.

$AsCl_4^-$ evidently corresponds to H_3O^+ in aqueous solutions and when the discussion is limited to $AsCl_3$ solutions we can therefore use the term Cl^- in the same way as we use H^+ in aqueous solutions.

Acids will decrease and bases will increase the Cl^- concentration in $AsCl_3$ (*cf.* the H^+ concentration in aqueous solutions).

Examples of acids are $FeCl_3$ and $SnCl_4$ which can form the ions $FeCl_4^-$ and $SnCl_6^{2-}$ and thus consume Cl^- ions.

A typical base is $(CH_3)_4NCl$ which as a salt contributes Cl^- ions to the solution. Another base to be discussed later is pyridine.

The new concept is more to be considered as a *method* for a *unified treatment* of what we call *acid-base reactions* in non-aqueous solvents and salt melts than as a contribution to the discussion of the *formal definition* of *acids and bases*. For that purpose there exist more symmetrical definitions [4] which, however, are purely formal.

It is of particular interest to compare the new concept with Lewis' theory, according to which an acid is an electron pair acceptor, a base is an electron pair donor. The difference in the two concepts is best shown in the two different ways of considering the reaction between pyridine and arsenic trichloride:

$$C_5H_5N: + AsCl_3 = C_5H_5N:AsCl_3 = C_5H_5N:AsCl_2^+ + Cl^-$$

The acid-base process is here, according to Lewis, the first step, that is *the formation of an electron pair bond*. That reaction would not change the Cl^- concentration in $AsCl_3$, however. The acid-base process described as *a transfer of chloride ions* would obviously be the second step.

The two definitions evidently have different aims. The reactions included in the Lewis theory are the same as those which cause the colour changes of indicators. The new concept on the other hand focuses interest on the transfer processes of ions, which can be studied indirectly with indicators but more directly with electrochemical methods.

THE EXPERIMENTAL STUDY OF IONIC TRANSFER PROCESSES

If the chloride ion transfer is the fundamental reaction in $AsCl_3$ there must exist $AsCl_2^+$ and $AsCl_4^-$ ions. The existence of such ions has only been proved indirectly and we have no knowledge of their structures.

The simplest indirect experimental evidence is obtained by preparations of compounds that may contain those groups. Thus the compound $AsSbCl_8$ has been interpreted as $AsCl_2SbCl_6$ [7], and the formula $(CH_3)_4NAsCl_4$ [7] as an indication of the presence of a $AsCl_4^-$ ion. These ideas have also been checked by conductometric neutralisation titrations [7], and must be considered to be well founded, although not conclusively so. Successive heating of the compound $(CH_3)_4NAsCl_4 \cdot 2\ AsCl_3$ also indicates that one $AsCl_3$ is more strongly bound than the two others [8].

If we thus confidently assume the existence of groups $AsCl_2^+$ and $AsCl_4^-$ it remains to be found whether there is any sense in speaking of a Cl^- concentration in $AsCl_3$. The existence of a pCl value is a condition for a successful quantitative application of the set of formulae already developed in the Brönsted theory to describe protolytic processes. In H_2O the pH values can be determined by electrochemical methods and the analogy would demand the possibility of determining pCl in a similar way. The problem is of course to find a reversible Cl^- sensitive electrode. AgCl is classified as very slightly soluble in $AsCl_3$ [7] and the possibility is then given of measuring concentration elements in $AsCl_3$. The cell would be

$$Ag,\ AgCl \mid Cl^-\ (C_1)\ in\ AsCl_3 \parallel Cl^-\ (C_2)\ in\ AsCl_3 \mid AgCl,\ Ag$$

Such measurements have been made and will be reported in a following paper [9]. It is a difficult task to determine Cl^- concentrations in this way because of the large activity correction connected with the low dielectric constants of $AsCl_3$. ($SeOCl_2$ would be a more suitable solvent for exact investigations.) The shape of titration curves, pCl (emf.) against acid added to a base, show, however, without doubt the analogy with pH measurements in water. It also proves the existence of a defined ionic product $C_{AsCl_4^+} \cdot C_{AsCl_4^-} = K$, characteristic for auto-ionized solvents.

Measurements of electrical conductivity have mostly been used in connection with conductometric titrations [7], but an effort has recently been made to prove the validity of the Onsager-Debye-Hückel theory for a strong electrolyte in $AsCl_3$ and to determine the acidity constants of some weak acids (V. Gutmann [10]). Determinations of transference numbers would also be of interest. Of course, measurements of all kinds of osmotic properties would give interesting information, too.

Studies of frequency shifts in As—Cl vibration spectra with different methods might give valuable indications about the degree of association in liquid $AsCl_3$ and about the interaction between acids or bases and $AsCl_3$.

COMPARISON WITH WATER AND AQUEOUS SOLUTIONS

The preliminary experimental investigations indicate the importance of chloride ion transfer in acid-base reactions in $AsCl_3$. The effect on pCl can be considered as a formal expression of an ionic transfer process but does not give any information about the mechanism of the transfer. An atomistic description of the constitution of $AsCl_3$ solution has still to be given. No structural work has as yet been done but a comparison with water and aqueous solutions may lead to a crude hypothetical picture apt to incite essential experimental studies.

The present state of knowledge about water and the compounds obtained from aqueous solutions is here briefly summarized for the purpose of comparison without any pretentions of completeness.

The structure of ice is well established. Each oxygen atom is surrounded tetrahedrally by four others. (This low coordination may be compared with 12 for dense packing.) The hydrogen atoms are distributed between the oxygen atoms, two hydrogen atoms being closely associated with each oxygen atom. The O—H...O bond is called a hydrogen bond.

When ice melts the lattice is partly broken down [11, 12], because part of the hydrogen bonds are broken [11, 12] or bent [13]. As a result of the partial dense packing, the average coordination number of oxygen is increased [11] from 4 to 4.9 at 80° C. The resulting differential increase of density is compensated at 4° C by the thermal expansion which makes the average distances longer. There is no doubt about the association of the water molecules to an ice-like structure, it may then be described as a "broken down ice structure" [11] or as a "network of pliable, branched chains of tetrahedra" [12].

The crystal structures of the compounds $HNO_3 \cdot H_2O$[14] and $HNO_3 \cdot 3\,H_2O$[15] show that there are hydrogen bonds between oxygen atoms in the nitrate groups and the water molecules. It has even been possible to prove the complete proton transfer from HNO_3 to H_2O in $HNO_3 \cdot 3\,H_2O$. The structure

suggested for aqueous HNO_3 solutions [16] is not quite in agreement with the structure found in the solid state. The discrepancy is probably due to the fact that Finbak [16] has overemphasized the importance of the tetrahedral coordination around the water molecules. In the solid state water molecules are found, which actually have triangular coordination [14, 15]. The assumption of an arrangement of the H_2O molecules around HNO_3 in such a way as to permit a proton transfer from HNO_3 to H_2O along hydrogen bonds is not influenced by this discrepancy. In dilute solutions of the strong acid HNO_3 the proton transfer is complete while only part of the protons are transferred from a weak acid to H_2O (or hydrogen bonds are formed with other acid molecules). The hydrogen bonds have of course a dynamical character in solutions, they are broken and redistributed and there is an average number of hydrogen bonds [12] determined by the temperature and by the nature and concentration of the solute.

In the same way NH_3 molecules form hydrogen bonds with the water molecules along which protons are partly transferred from H_2O to NH_3 forming NH_4^+ and OH^- ions.

An addition of acid evidently results in an excess of protons in the water structure, symbolized as H_3O^+. In a similar way a base gives rise to a proton deficiency symbolized as OH^-. The pH value of a solution is dependent on this proton excess or proton deficiency.

The arrangement of water molecules around ions which do not function as acids or bases has been extensivily studied in the solid state structures of salt hydrates. It has been shown in an excellent review [17] that the water molecule does not exhibit any tetrahedral character in such compounds but demonstrates one positive and one negative side. The hydration of ions in aqueous solutions is usually explained in a similar way. In the case of large ions with small charges, such as Cs^+, the water molecules around the ion will still belong to the water structure. With decreasing size or increasing charge hydrated ions such as $Mg(H_2O)_6^{2+}$ are formed. A further change will lead to a proton repulsion as in the case of Al^{3+}, causing the acidic properties of an $AlCl_3$ solution.

It seems as if proton transfer is intimately connected with the existence of hydrogen bonds along which the transfer can take place without any other structural changes. There are, however, acid-base reactions which involve drastic structural changes: $7\ MoO_4^{2-} + 8\ H^+ = Mo_7O_{24}^{6-} + 4\ H_2O$.

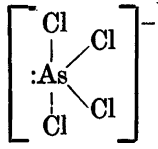
The existence of hydrogen bonds may be considered as due to the smallness of the proton which makes any other oxygen coordination than two around the protons very little probable. The hydrogen bond thus cannot be expected necessarily to have an analogy in other ionic transfer processes.

As a basis for discussion of the constitution of $AsCl_3$ solutions we have no more knowledge than of the structure of the free $AsCl_3$ molecule [18], a pyramid with the As—Cl distances 2.16 ± 0.03 Å and the Cl—As—Cl angles $103 \pm 3°$. It can also be described as a slightly distorted tetrahedron with one vacant position. The cohesive forces in the solid $AsCl_3$ are probably determined by the interaction between such molecules. It would therefore be interesting to know the coordination around an $AsCl_3$ molecule and whether the structure is such as to permit the existence of chlorine bonds and thus ionic transfer (at the autoionization) without any other structural changes. It is easy to derive such

structures making different assumptions, but it is not worth-while to discuss them all because they are quite arbitrarily obtained. To demonstrate the principles one single case will be mentioned, namely the formation of chains of distorted $AsCl_4$ tetrahedra in the structure. That would include chlorine bonds from one arsenic atom to the other

$$\begin{matrix} & Cl & & Cl & & Cl & \\ \ldots Cl- & As & \ldots Cl- & As & \ldots Cl- & As & . \\ & Cl & & Cl & & Cl & \end{matrix}$$

Formation of $AsCl_2^+$ and $AsCl_4^-$ would then be possible by a simple transfer process without any further structural changes.

For many reasons this hypothesis is not very probable. It is recognized that the lone electron pair in $AsCl_3$ is of importance for the structure, and it is often arbitrarily assumed to be situated in the fourth corner of the tetrahedron Cl
:As—Cl. In the same way the structure of $AsCl_4^-$ would be determined by the Cl
following electron distribution  and thus have a trigonal bipyr-

amidal structure with one vacant position and not a tetrahedral structure.

Only structure determinations can give a definite answer to this problem and thus indirectly to the question how a chloride ion excess or deficiency is distributed in the liquid structure.

Comparison with the structures of some other solid non-aqueous solvents supports, however, the idea that chlorine bonds do not exist. If the structure of the solids is mainly preserved in the liquid state ionic transfer without other structural changes is not possible in SO_2[19] or $COCl_2$[20].

A consideration of the solution of $SnCl_4$ in $AsCl_3$ will give further aspects of the problem. The $SnCl_4$ molecule has a tetrahedral structure and must increase its coordination to an octahedral structure in the acid-base reaction $SnCl_4 + 2AsCl_3 = SnCl_6^{2-} + 2AsCl_2^+$. The chloride ion transfer here is connected with a complete change in the distribution of the chloride ions around the Sn atoms. Of course there is a possibility that an intermediate compound $SnCl_4(ClAsCl_2)_2$ exists with an octahedral coordination. This possibility has a perfe ct analogy in aqueous solutions: $CO_2 + H_2O = H_2CO_3 = H^+ + HCO_3^-$.

Evidently such molecules or ions will function as acids which have a chloride ion affinity. This may partly be determined by purely geometrical considerations. The low chloride ion affinity of bases can in some cases be explained as a repulsion effect. There is thus an analogy between the two reactions:

$$C_5H_5NAsCl_3 = C_5H_5NAsCl_2^+ + Cl^- \quad \text{(N—Cl repulsion)}$$

and

$$Al(OH_2)_6^{3+} = Al(OH)(OH_2)_5^{2+} + H^+ \quad \text{(Al—H repulsion)}$$

Whatever is the mechanism of the ionic transfer processes in $AsCl_3$, it will result in chloride ion excess or deficiency, which can be measured.

All the problems discussed here are evidently very much dependent on the charge distribution in $AsCl_3$. Ions such as $(CH_3)_4N^+$ are probably surrounded by $AsCl_3$ molecules turning their negative sides against the positive ion. The knowledge of the structures of solvates is thus of fundamental importance for any effort to discuss solvation processes or to obtain a picture of the structure of solutions in $AsCl_3$.

PERTINENT STRUCTURAL PROBLEMS

An attempt has been made to show that our present knowledge permits only vague ideas about the constitution of $AsCl_3$-solutions and to point out some pertinent structural problems which are here summarized:

1. The structure of solid and liquid $AsCl_3$ is of basic importance in any structural discussion of $AsCl_3$ solutions.

2. The structures of the ions $AsCl_2^+$ and $AsCl_4^-$ might explain how Cl^- excess or deficiency is manifested in the liquid structure.

3. The knowledge of the structures of solid solvates is necessary for any detailed discussion of solubility and solvation conditions in $AsCl_3$-solutions.

Of course these points apply directly to the study of other auto-ionizing solvents.

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Studies on Arsenic Trichloride as a Solvent

III. Potentiometric Acid-base Titrations in $AsCl_3$

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Potentiometric titrations have been carried out in $AsCl_3$ solutions using Ag, AgCl electrodes. The idea of a Cl^- ion concentration governing the acid-base processes has been confirmed by these measurements.

In the preceding paper [1] the importance of potentiometric measurements of pCl in $AsCl_3$ solutions was pointed out and the use of Ag, AgCl-electrodes was suggested. This paper will deal with the possibilities of measuring large pCl changes in potentiometric acid-base titrations in $AsCl_3$. The definition acid $+ Cl^- =$ base leads to the use of the following concentration cell:

$$Ag,\ AgCl \mid Cl^-\ (C_1) \parallel Cl^-\ (C_2) \mid Ag,\ AgCl$$

Titrations have been carried out with the acid $FeCl_3$ against the bases $(CH_3)_4NCl$, C_5H_5N, $(C_2H_5)_2NH$ and with the base $(CH_3)_4NCl$ against the acids $FeCl_3$ and $SbCl_5$.

A comparison with a conductometric titration has also been made.

EXPERIMENTAL

Chemicals used. Commercial $AsCl_3$ was purified by two distillations at a final boiling point of 229° C. The conductance of the distilled $AsCl_3$ was measured in the part A of the receiver (Fig. 1). (The instrument used was a Philips Philoskop, type GM 4140, and the measurements were performed at a frequency of 1 000 p/s.) As long as the conductance was considered to be too high the liquid was poured out through K to B. When the purity was satisfactory the liquid was permitted to pass through the syphon S to C. Table 1 shows the results of one such distillation. The fractions following 7 gave the same conductance and were all collected in the flask C. The conductance was constant after 12 hours. The lowest value [2] previously measured in $AsCl_3$ seems to be $1.4-1.6 \cdot 10^{-7}$ ohm^{-1} cm^{-1} at 0° C. Our value obtained at + 19° C is considerably lower, although the conductance of $AsCl_3$ has a positive temperature coefficient [2].

The other chemicals were all purified by standard methods, with particular care to keep them dry.

Electrodes. The Ag, AgCl electrodes were made according to Brown [3]. Before use they were left in ethyl ether for 24 hours and then heated to 130° C during one hour.

The design of the cell is shown in Fig. 2 a. The reference electrode R is filled with a solution of $(CH_3)_4NCl$ in $AsCl_3$. The carefully ground-in stopper at J still permits an equilibrium potential to be obtained. Emf was measured with a vacuum tube voltmeter (*Ermi*).

Apparatus. Preliminary experiments showed that it was most unsatisfactory to use stopcocks in the apparatus because of the creeping out of the $AsCl_3$ solution and because of chemical reactions with every available type of grease. A special titration apparatus was therefore designed to avoid stopcocks or any contact between solution and greased parts (Fig. 2 a and b). The reagent solution is passed from the vessel V into the U-tube system consisting of tubes A and B. The addition of reagent to the solution in K takes place in the following way. The whole apparatus, Fig. 2 a, is tilted clockwise in the plane of the paper. The buret is then turned around S so that the solution will rise in A. The solution will begin to flow from A to C at about 90° rotation and the bent tip at T will then point downwards allowing a drop-wise addition of solution. (The amount of solution in A—B must be such that it can be completely transferred to A.) The apparatus could readily be manipulated in such a way that the solution in C would flow back into A—B. By means of a calibrated scale attached to A—B volume readings were taken before and after the addition of solution through C. During the conductometric titration a conductance cell (of the same shape as shown in Fig. 1) replaces the electrode system.

Results. The results are summarized in Table 2 and Figures 3—5.

DISCUSSION OF THE RESULTS

The ionic character of the reactions studied here has previously been established by conductometric titrations [4]. The fact that the potentiometric titrations give as satisfactory results as indicated by Table 2 shows further,

Table 1.

Fraction no.	Spec. cond. ohm^{-1} cm^{-1}
1	$5.7 \cdot 10^{-6}$
2	$1.8 \cdot 10^{-6}$
3	$1.0 \cdot 10^{-6}$
4	$5.6 \cdot 10^{-7}$
5	$2.8 \cdot 10^{-7}$
6	$1.2 \cdot 10^{-7}$
7	$1.0 \cdot 10^{-7}$

Table 2.

Solution in K		End.-point (ml)		Solution in V		Fig. no.
Solute	mmole solute / 1 000 g $AsCl_3$	Calc.	Found	Solute	mmole solute / 1 000 g $AsCl_3$	
$(CH_3)_4NCl$	0.21	2.55	2.56	$FeCl_3$	1.4	3 a
C_5H_5N	0.50	6.36	6.54	$FeCl_3$	1.4	3 b
$(C_2H_5)_2NH$	0.3	2.2	2.34	$FeCl_3$	1.4	3 c
$FeCl_3$	0.26	2.69	2.68	$(CH_3)_4NCl$	1.7	4 a
$SbCl_5$	0.47	2.96	3.01	$(CH_3)_4NCl$	1.7	4 b
$(CH_3)_4NCl$	0.29	4.38	4.39	$FeCl_3$	1.4	5

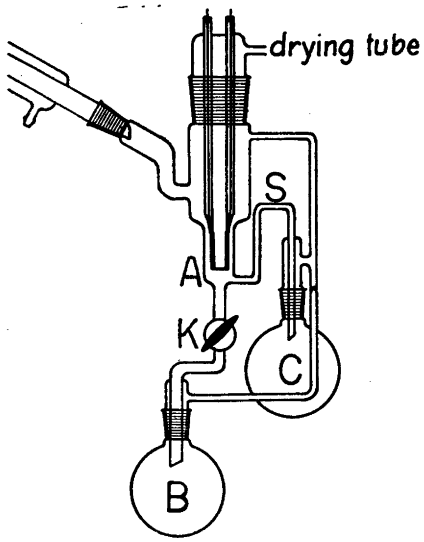

Fig. 1. Reception part of the apparatus used for purification of $AsCl_3$ by distillation.

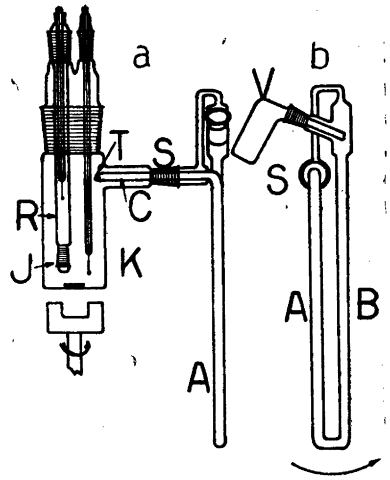

Fig. 2 a. Titration apparatus. b. Perpendicular view of the buret.

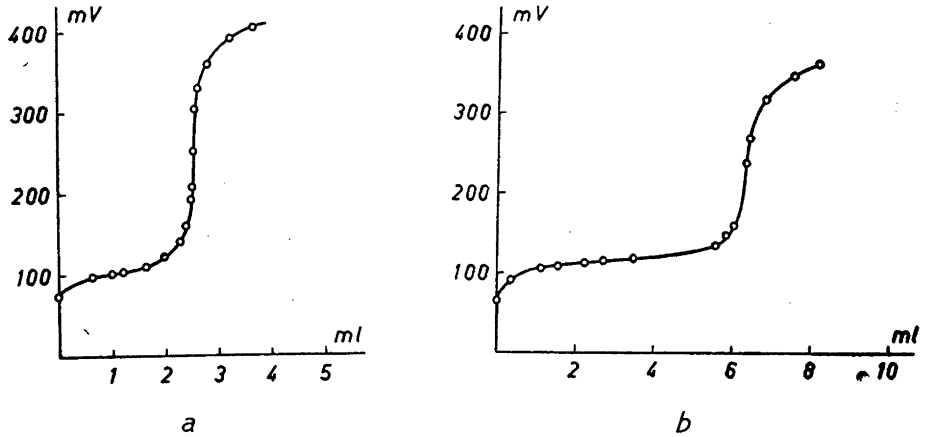

Fig. a. b. Potentiometric titration curves (see Table 2).

that the Ag, AgCl electrode behaves at least roughly as a pCl electrode. There is thus reason to speak of a Cl^- concentration in the $AsCl_3$ solution and to write, *e.g.*, the reaction between $(CH_3)_4NCl$ and $FeCl_3$ as

$$Cl^- + FeCl_3 = FeCl_4^-$$

The existence of an ionic product and of a scale of acid and base strength follows from the confirmation of the existence of a Cl^--concentration. The former may be best studied by conductometric methods [5].

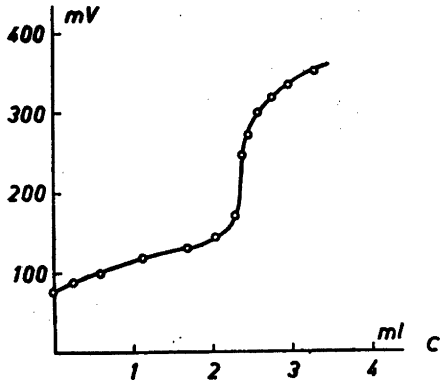

Fig. 3 c. Potentiometric titration curves (see Table 2).

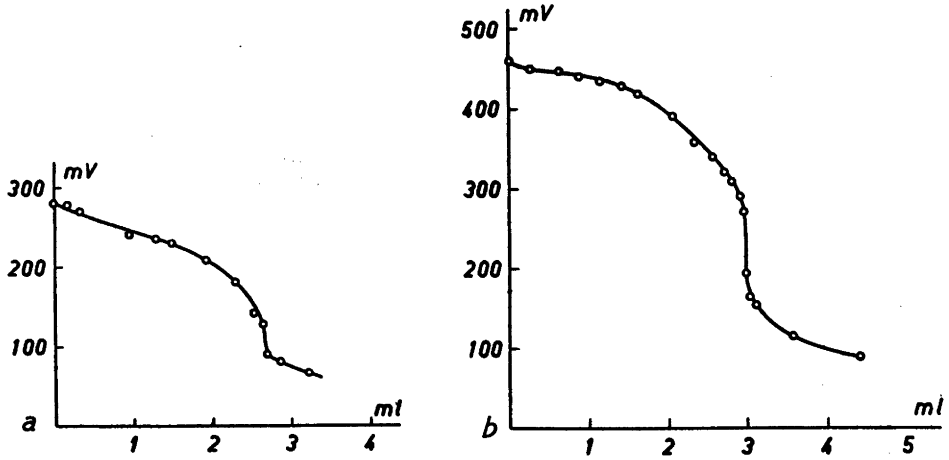

Fig. 4. Potentiometric titration curves (see Table 2).

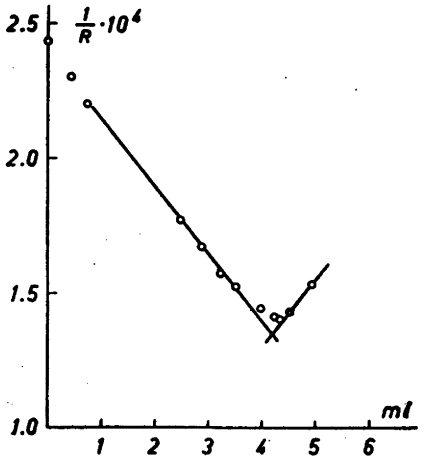

Fig. 5. Conductometric titration curve (see Table 2).

A maximum value can be obtained for the product $K = C_{AsCl_2}^{+} \cdot C_{AsCl_4}^{-}$ if we assume that the liquid junction potential can be neglected and that $(CH_3)_4NCl$ as well as $SbCl_5$ are strong electrolytes in $AsCl_3$. We obtain from Fig. 4 b

$$^1/_2\ pk = \frac{460—215}{60} - \log C_{SbCl_5},$$

giving $pk > 15$ and $C_{AsCl_2}^{+} \cdot C_{AsCl_4}^{-} < 10^{-15}$.

We cannot hope to come further with the potentiometric methods without elaborate experiments and extended theoretical calculations. There are different reasons to expect difficult problems:

1. The unknown liquid junction potential.
2. The unknown degree of dissociation of the acid and base solutions in $AsCl_3$.
3. The unknown acid-base behavior and solubility product of the slightly soluble AgCl in $AsCl_3$.
4. The difficulties involved in determining the activity factors in solvents with low dielectric constants.

These problems are too complex to give much hope of a very accurate treatment. We intend, however, to explore further the possible usefulness of the potentiometric methods for studying ionic reactions in $AsCl_3$ and other solvents.

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The Alkylation of Lignin with Alcoholic Hydrochloric Acid

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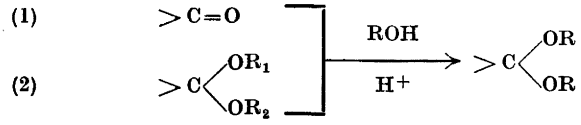
Brauns' "native lignin" from spruce was shown to contain 0.12 CO groups per methoxyl. By reduction with sodium borohydride a practically carbonyl-free preparation was obtained. The original as well as the reduced lignin were subjected to treatment with 0.5 % methanolic hydrochloric acid either during 48 hours at room temperature or during 2.5 hours under reflux. Extensive methylation took place in each case, the ratio OCH_3 introduced/OCH_3 orig. present ranging from 0.41 to 0.52.

From these results it is concluded that the methylation is not due to acetal formation. The most probable explanation is the etherification of benzyl alcohol groups and the re-etherification of benzyl ether groups present in "native lignin".

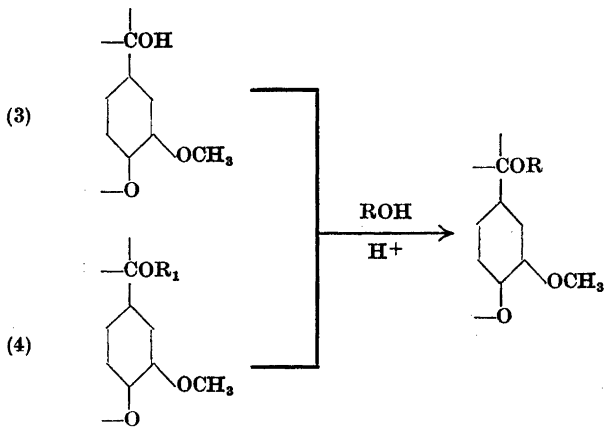
In 1893, Klason [1] made the observation that some lignin was brought into solution when spruce wood was heated with ethanol containing a small amount of hydrogen chloride. This reaction has been studied more closely by Holmberg [2,3], who found that the formation of the "ethanol lignin" involves the uptake of approximately 0.5 ethoxyl groups for every methoxyl present in the lignin. Several alcohol lignins have been prepared from wood by using alcohols other than ethanol, and in all cases alkylation of the lignin was demonstrated [4,5]. Similarly, "native lignin", isolated according to Brauns' method, *i. e.*, a lignin fraction which is extracted from wood by ethanol at room temperature and which amounts to a few per cent of the total lignin, is methylated when heated with methanolic hydrochloric acid [6].

This alkylation of lignin has been interpreted mainly in two ways. Holmberg and Runius [2] assumed that lignin contains carbonyl groups partly involved in an acetal-like linkage between lignin and carbohydrates. Heating with alcoholic hydrochloric acid was then believed to bring about an acetalization (1) of free carbonyl groups or a re-acetalization (2) of bound carbonyl groups.



This view, which was shared by several other authors, seemed to find support in the fact that the alkoxyl groups were removed when the alcohol lignin was treated with strong acids [7].

An alternative explanation was suggested by Holmberg [8] in 1935. On the basis of experiments with model substances, such as α-phenyl ethyl alcohol and benzhydrol, he assumed the presence in lignin of exceedingly reactive alcoholic hydroxyl or ether groups, *viz.*, benzyl alcohol or benzyl ether groupings. The alkylation reaction was then considered as an etherification of benzyl alcohol groups (3) or a re-etherification of benzyl ether groups (4), or both.



The same types of groups were assumed to be responsible for other typical lignin reactions such as the sulphonation with sulphite solutions and the formation of lignothioglycolic acids.

Concerning the two last-mentioned reactions, and especially the sulphonation, Holmberg's benzyl alcohol and benzyl ether hypothesis has been supported by a vast amount of experimental work (*cf.* Lindgren [9]).

The alkylation of lignin, however, has been comparatively little studied and, until recently, has been interpreted in the sense of Holmberg's older theory. Thus, Brauns expressed the view that the methylation, which takes place when isolated "native lignin" is heated with methanolic hydrochloric acid, is probably due to the acetalization of carbonyl groups [6, 10]. It is known, however, that *p*-hydroxybenzyl alcohols are readily etherified by alcoholic hydrochloric acid, and also that corresponding benzyl alkyl ethers are re-etherified under very mild conditions [11]. With this fact in mind it was attempted to find conclusive evidence for the mechanism of the alkylation reaction.

The lignin material used in the present work was „native lignin" isolated according to Brauns [6] from spruce (*Picea excelsa*). The preparation contained 14.67 % OCH_3. By potentiometric titration of the hydrochloric acid which was liberated when the lignin was treated with hydroxylamine hydrochloride, the preparation was found to contain 1 CO group per 8.3 OCH_3 groups ($CO/OCH_3 = 0.12$). Since "native lignin" contains roughly one coniferyl aldehyde group per forty methoxyl groups [12], about every tenth guaiacyl

propane monomer thus carries a carbonyl group not belonging to a coniferyl aldehyde structure.

On treatment of the "native lignin" with sodium borohydride in aqueous ethanol, a reduced "native lignin" with a methoxyl content of 15.19 % was obtained, in which only traces of carbonyl groups (about 3 % of the amount originally present) could be detected (see Fig. 4). The presence of carbonyl groups in the original preparation and their elimination on sodium borohydride reduction was also demonstrated by infrared absorption measurements *. Whereas the original "native lignin" shows a definite band in the C=O region (about 1 660 cm^{-1}), only a slight inflection is observed at this position in the absorption curve of the reduced lignin (Fig. 1, a and b).

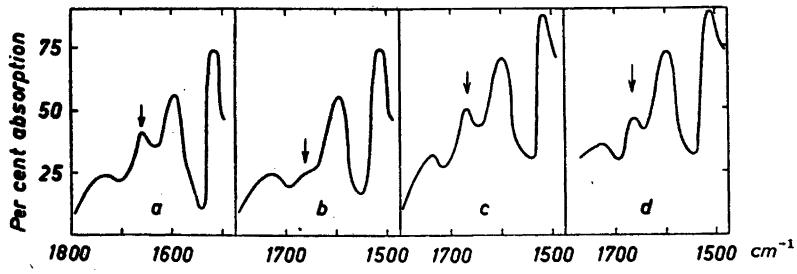


Fig. 1. Infrared absorption curves. a. "Native lignin". b. "Native lignin", reduced with sodium borohydride. c. "Native lignin", methylated with 0.5 % $CH_3OH-HCl$ (48 h, room temp.). d. "Native lignin", methylated with 0.5 % $CH_3OH-HCl$ (2.5 h, reflux). The arrows mark the position of the C=O absorption.

Fig. 2 shows the ultraviolet absorption curves of the original and the reduced "native lignins". The reduced product gives a considerably lower absorption in the range above 300 mμ compared with the original preparation. The decrease must be partly due to the reduction of coniferyl aldehyde residues, which cause a comparatively high extinction in this wavelength range [13], yielding coniferyl alcohol groups. This is also indicated by the fact that the reduced product does not give the colour reaction with phloroglucinol and hydrochloric acid, in contrast with the original preparation [14]. Further, the nearly white colour of the reduced product suggests that quinoid groupings have been eliminated.

The original as well as the reduced lignin were subjected to methylation with methanol containing 0.5 % hydrogen chloride, *i. e.*, the reagent used by Brauns [6] in the methylation of "native lignin" from black spruce *(Picea mariana)*. In Brauns' experiments the reaction mixture was heated under reflux for 2.5 hours. It was found, however, that treatment at room temperature during 48 hours was sufficient to produce extensive methylation, and therefore the experiments were carried out both at room temperature (48 hours) and under reflux (2.5 hours).

* The authors are indebted to Dr. K. Almin for kindly carrying out the infrared measurements.

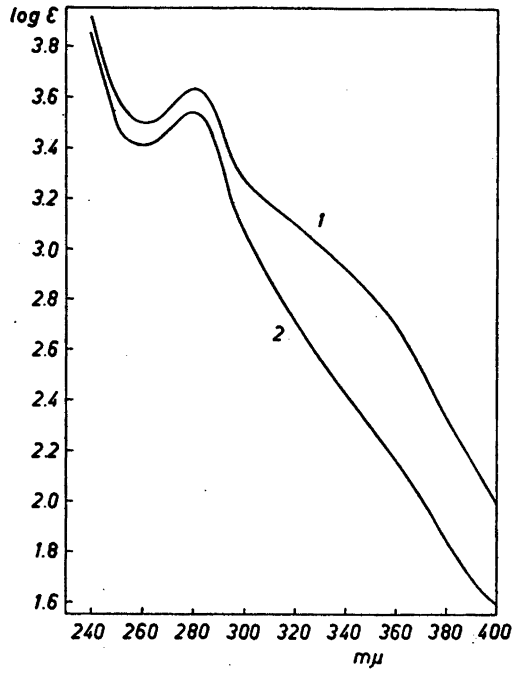


Fig. 2. Ultraviolet absorption curves. Curve 1: "Native lignin". Curve 2: "Native lignin", reduced with sodium borohydride. Based on methoxyl concentration. Solvent: 96 % ethanol.

The ultraviolet absorption curves of the resulting methylated products (Fig. 3) do not reveal any distinct structural changes due to the methylation when compared with the absorption of the corresponding starting materials (Fig. 2).

The analytical findings are summarized in Table 1. From the increase in the methoxyl content after methylation at room temperature, it can be calculated that the original "native lignin" acquired 0.47 new OCH_3 groups for

Table 1.

Lignin preparation	Before methylation % OCH_3	After methylation at room temp., 48 h		After methylation at reflux, 2.5 h	
		% OCH_3	$\frac{OCH_3 \text{ introd.}}{OCH_3 \text{ orig.pres.}}$	% OCH_3	$\frac{OCH_3 \text{ introd.}}{OCH_3 \text{ orig.pres.}}$
"Native lignin"	14.67	20.49	0.47	21.09	0.52
Reduced "native lignin"	15.19	20.46	0.41 [0.47]	21.02	0.46 [0.51]

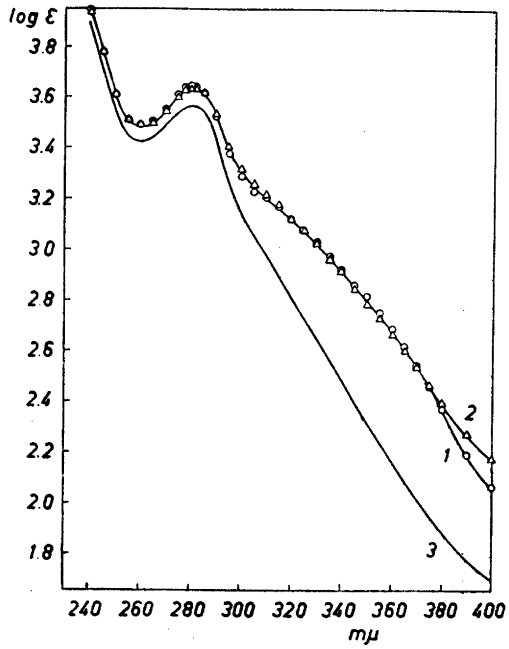


Fig. 3. Ultraviolet absorption curves. Curve 1: "Native lignin", methylated with 0.5 % $CH_3OH-HCl$ (48 h, room temp.). Curve 2: "Native lignin", methylated with 0.5 % $CH_3OH-HCl$ (2.5 h, reflux). Curve 3: Sodium borohydride-reduced "native lignin", methylated with 0.5 % $CH_3OH-HCl$ (2.5 h, reflux). All curves based on concentration of OCH_3 present in unmethylated starting material. Solvent: 96 % ethanol.

every OCH_3 originally present. The corresponding value, 0.52, obtained when the reaction was performed under reflux, is in good agreement with the result reported by Brauns [6] in the case of black spruce "native lignin".

The most important finding shown in Table 1 is that the reduced lignin preparation behaves similarly to the original lignin. The methoxyl values of the methylated reduced preparations are practically identical with those of the methylated non-reduced lignins.

Due to the fact that the methoxyl value of the reduced lignin (15.19 %) is slightly higher than that of the original lignin (14.67 %), the ratio OCH_3 introduced/OCH_3 orig. present becomes somewhat lower in the first case than in the latter one. One might assume that the comparatively high methoxyl value of the reduced lignin is caused by an etherification of some highly reactive hydroxyl groups by the ethanol used as a solvent in the sodium borohydride reduction and also present during the recovery of the reduced product. Unintentional temporary acidification after completed reduction (*cf.* experimental part) might be sufficient to bring about such slight ethylation. If this assumption is accepted, the alkylation values given in brackets are obtained. These "corrected values" are nearly identical with those obtained in the methylation of the non-reduced lignin.

But even if no consideration is taken of such a correction, it is obvious that methylation takes place to a very similar extent irrespective of the presence or

absence of carbonyl groups in the lignin material. Hence, the methylation cannot be due to acetal formation.

This conclusion is further supported by the fact that the carbonyl band of the infrared absorption curve of "native lignin" does not disappear on methylation (see Fig. 1, c and d) *. Obviously, the carbonyl groups present in spruce "native lignin" do not form stable acetals. If there is any acetalization during the interaction of the methanolic hydrochloric acid, the acetals or ketals formed must be hydrolyzed again during the isolation of the methylated product. It may also be noted that the carbonyl groups present in the original "native lignin" (0.12 CO/OCH_3), if completely acetalized, could give rise to at most 0.24 $OCH_{3\ introduced}$ per $OCH_{3\ orig.\ present}$, whereas the figures actually obtained are 0.47 and 0.52.

The question now arises which types of groups are responsible for the methylation.

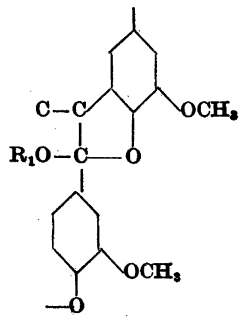
It was found that the methoxyl content (20.1 % OCH_3) of a methylated product obtained by treatment of "native lignin" at room temperature for 24 hours with 0.5 % methanolic hydrochloric acid, remained practically unchanged in 0.1 *N* aqueous sodium hydroxide (48 hours, room temperature), the recovered product containing 19.8 % OCH_3. This result shows that the introduced methoxyl groups are not present as ester groups. It is in agreement with the alkali stability of the methoxyl and ethoxyl groups in methanol and ethanol lignins from spruce wood as reported by Hibbert and Brauns [7], and by Holmberg and Runius [2].

Since acetalization and ester formation can be excluded, the alkylation must be due to the reaction of hydroxyl groups in the lignin. There are three possible types of such groups which are sufficiently reactive to undergo alkylation with alcoholic hydrochloric acid.

One type to be considered is the α-ketol structure, the hydroxyl group of which is known to be etherified by alcoholic hydrochloric acid [16]. It is also known by the work of Hibbert [17] that monomeric α-ketol ethers are formed if wood or isolated lignin is heated for 48—72 hours with ethanolic hydrochloric acid. The appearance of side-chains with an α-ketol structure with subsequent etherification of its hydroxyl group during the treatment of native lignin with methanolic hydrochloric acid might therefore be considered possible. In this case, the alkylation of lignin would be expected to be accompanied by the formation of keto groups. By means of the hydroxylamine hydrochloride method it was found, however, that sodium borohydride-reduced and subsequently methylated "native lignin" (methylation during 2.5 hours under reflux) was practically free of carbonyl groups. Hence, the methoxyl groups introduced into the lignin are not present as α-ketol ether groupings.

Some years ago, Erdtman [18] suggested the presence of cyclic hemiacetal structures (I) in lignin. The open hydroxyl group (OR_1, R_1 = H) or an alkyl derivative of it (—OR_1) would be the site of the typical lignin reactions such as sulphonation and alkylation with alcoholic hydrochloric acid. Sulphonation

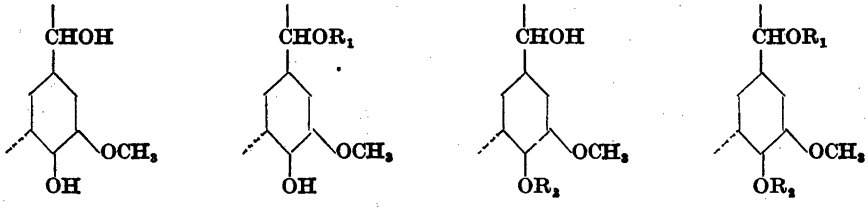
* The opposite observation has been reported by Jones [15], who states that the absorption band in the spectrum of black spruce „native lignin" at 1 663 cm^{-1} is no longer found in the spectrum of the CH_3OH—HCl methylated lignin. The reason for this discrepancy is obscure.



I

studies with a model substance [18] containing a structure not very closely related to I constitute the only experimental support hitherto available, and further model experiments would be highly desirable. Although, at present, the possibility of structures like I being responsible for the alkylation and other reactions of lignin cannot be excluded, it appears that its further discussion must be postponed until more experimental material has been collected.

The benzyl alcohol and benzyl ether concept of lignin structure seems to provide an excellent basis for the explanation of the alkylation with alcoholic hydrochloric acid. In addition, the results reported in the present paper strongly support this concept. The following structures, *a*—*d*, previously suggested to be present in lignin of coniferous wood mainly as a result of sulphonation studies (*cf.* Ref. [9]), can undergo alkylation in the benzyl position:



a *b* *c* *d*

The *p*-hydroxybenzyl alcohol structure *a* and the *p*-alkoxybenzyl alcohol structure *c* will be alkylated by etherification, whereas the *p*-hydroxybenzyl (alkyl) ether structure *b* and the *p*-alkoxybenzyl (alkyl) ether structure *d* will react by re-etherification.

Direct experimental evidence for the occurrence in lignin of the phenolic structures *a* and *b* has recently been provided. Thus, the "quinone methide reaction" of "native lignin", briefly reported by Adler and Gierer [19], is probably due to the presence of one or both of these structures, and the „indophenol reaction" of wood and isolated "native lignin", which has been described by Gierer [20], has to be ascribed to structure *a*.

The extent of methylation (about 0.5 OCH_3 introduced / OCH_3 orig. present) indicates that the methylatable structures *a*—*d* constitute together about

50 % of the guaiacyl propane monomers of spruce "native lignin". This, of course, is true only if not more than one OCH_3 is introduced into each of the methylatable monomers. According to Gierer [20] structure *a* occurs in 12—14 % of the monomers.

If the carbonyl groups in "native lignin" (0.12 CO per OCH_3), apart from those belonging to coniferylaldehyde residues (about 0.025 per OCH_3), were keto groups in the α-position of the side-chain, *i. e.*, adjacent to the aromatic nucleus, sodium borohydride reduction of the lignin would be expected to produce about 0.1 new methylatable aryl carbinol group of the types represented by structures *a* and *c*. However, the methylation values of the reduced lignin are not higher than those of the original preparation. This seems to indicate that the carbonyl groups are mainly not in the α-position of the side-chains. In agreement herewith, Gierer [20] arrived at the conclusion that phenolic units with α-keto groups seem not to be present in "native lignin", since the amount of groups *a*, estimated by the „indophenol reaction", was not increased after reduction of the lignin with sodium borohydride.*

It can be expected that the four structures *a—d* will differ in their reactivity towards alcoholic hydrochloric acid. In fact, model experiments, which are still under progress [21], have shown that *p*-hydroxybenzyl alcohols (type *a*) react faster than *p*-alkoxybenzyl alcohols (type *c*), and, in addition, there are certain differences in reactivity between free benzyl alcohols (*a*, *c*) and benzyl ethers (*b*, *d*). Furthermore, sharp dissimilarities have been found in the behaviour of different benzyl ethers. Whereas ethers, in which R_1 (in formulae *b* and *d*) is an open alkyl residue, are readily re-etherified, cyclic ethers such as dehydro-di*iso*eugenol and pinoresinol are more or less resistant to methanolic hydrochloric acid under the conditions used in the present investigation. Finally, it has been found that the course of the alkylation of aryl propane derivatives containing the structures *a—d* is greatly dependent upon the chemical nature of the substituents present at the β- and γ-positions of the propane side-chain. Thus, a closer examination of the alkylation of lignin, in comparison with the behaviour of appropriate model substances, seems to offer a new means of elucidating the principles of lignin structure. In this connection, the finding that alkylation takes place under very mild conditions is of considerable interest.

EXPERIMENTAL

Reduction of "native lignin" with sodium borohydride. The procedure has already been described in a preceding paper by Gierer [20]. In the present case, the reduction was carried on for two days at room temperature, and the alkaline reaction mixture neutralized with hydrochloric acid.

Estimation of carbonyl groups. Brauns' "native lignin" [6] from spruce wood meal or the sodium borohydride-reduced product (100 mg) was dissolved in 25 ml 96 % ethanol. The pH of the solution was adjusted to 3.5 by the addition of dilute aqueous hydrochloric acid. A solution of 0.17 g NH_2OH-HCl in 10 ml ethanol, likewise adjusted to pH 3.5, was added. A stream of nitrogen was bubbled through the mixture, which after certain

* *Note added in proof:* In a recent publication a similar result was reported by G. Aulin-Erdtman [*Svensk Papperstidn.* **57** (1954) 745], who found by spectrophotometric measurements on different lignin preparations that the sum of α-keto groups and C—C double bonds conjugated with the aromatic ring can not be greater than 0.01—0.02 per OCH_3.

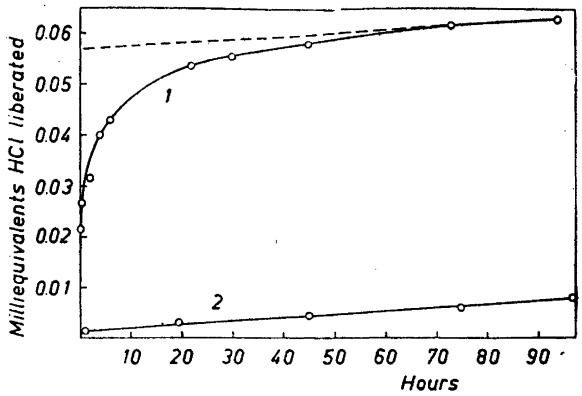


Fig. 4. Estimation of carbonyl groups. Curve 1: "Native lignin". Curve 2: Sodium borohydride-reduced "native lignin".

time intervals was titrated with 0.0099 *N* NaOH to pH 3.5 (glass electrode). The consumption of sodium hydroxide is shown in Fig. 4. The slow liberation of hydrochloric acid indicated by the nearly linear slope of the right part of the curves is caused by some oxidative decomposition of hydroxylamine, due to incomplete exclusion of air. The true consumption of sodium hydroxide due to oxime formation was found by extrapolation to time zero.

"Native lignin" (curve 1): 100 mg lignin with 14.67 % OCH_3, *i.e.*, 0.47 mequ. OCH_3, consumed 0.057 mequ. NaOH, corresponding to 0.057 mequ. CO. $CO/OCH_3 = 0.12$.

The reduced lignin (15.19 % OCH_3) consumed about 0.002 mequ. NaOH (curve 2). $CO/OCH_3 = 0.004$.

Methylation with 0.5 % methanolic hydrochloric acid. The procedure was similar to that already reported by Gierer [20]. The lignin preparation (200 mg) was dissolved in 4 ml absolute methanol, and 1 ml methanol containing 0.25 g hydrogen chloride was added. The mixture was either allowed to stand at room temperature for 48 hours or refluxed for 2.5 hours, then neutralized with a few drops of 2 *N* Na_2CO_3 solution and evaporated *in vacuo* to dryness. The residue was dissolved in 2 ml absolute methanol-acetone mixture (1 : 1), filtered, and washed with a few ml of the same solvent. The solution was poured into 60 ml of water. The precipitate, after standing overnight, was collected on a glass filter, washed thoroughly with water and dried *in vacuo* over phosphorus pentoxide. The yield of this product was about 60 %. It was dissolved in dioxan and re-precipitated in ethyl ether [5]. Yield about 50 % of the original lignin.

The calculation of the ratio OCH_3 introduced/OCH_3 orig. present may be illustrated by the following example:

Methylation of "native lignin" in boiling CH_3OH—HCl, 2.5 hours. "Native lignin", 14.67 % OCH_3; methylated product, 21.09 % OCH_3.

If methylation is assumed to involve the etherification of hydroxyl groups, the increase in weight is $CH_2 = 14$ for every introduced methoxyl group. If it is assumed to involve the re-etherification of ether bridges, the corresponding increase is $CH_3OH = 32$. The mean value of 23 has been adopted for the present calculation.

The two equations

$$\frac{31.034 \cdot 100}{x} = 14.67,$$

and

$$\frac{(1 + y) \cdot 31.034 \cdot 100}{x + y \cdot 23} = 21.09$$

give x (= the weight of one OCH_3 equivalent in "native lignin") = 211.5, and y (= OCH_3 introduced/OCH_3 orig. present) = 0.52.

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The Catalatic Decomposition of Hydrogen Peroxide and Its Retardation by Azide

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It is confirmed that at low concentrations of hydrogen peroxide its catalatic decomposition follows a first order law. A linear relationship between the reciprocal rate constant and the concentration of azide ion is found. From the experiments the equilibrium constant for the equilibrium between an inactive catalase-azide complex and active catalase is determined.

Zeile and Hellström [1] have shown that catalase is a hemoprotein in which the hematin acts as a prostetic group.

In the present work horse kidney catalase is used. According to Bonnichsen [2] it has four hemine groups per molecule, while horse liver catalase contains three hemines and one verdohemochromogen group.

Several theories have been advanced to explain the decomposition of hydrogen peroxide by catalase. Theorell [3] and Chance [4] both find that two hydrogen peroxide molecules react with one catalase molecule. According to Theorell each hydrogen peroxide molecule is attached to one hematin group and from two such groups oxygen and water are subsequently released. Chance, however, assumes that one hydrogen peroxide molecule is bound to one hematin group and that this group then reacts with a free hydrogen peroxide molecule.

The decomposition of hydrogen peroxide by means of catalase is a first-order reaction provided the catalase concentration is about 10^{-9} M [6]. Svendsen [7] has found that a first-order reaction is also obtained if the catalase concentration is about 10^{-10} M provided the substrate concentration is low.

Experiments at low hydrogen peroxide concentrations (*ca.* 10^{-3} M) were carried out and confirmed that the enzyme under these circumstances is not (or at least only very slowly) inactivated by the hydrogen peroxide: The first-order reaction constant was determined from 4—5 titrations during a time corresponding to one or two half-periods. After a further 5—6 half-periods fresh hydrogen peroxide was added to make the concentration up to about the original and measurements were again taken over about two half-periods to give a new determination of the rate constant. Most frequently the two

determinations were identical; still it happened that the last was only say eighty per cent of the first. But according to Svendsen [8] this decrease in activity is probably not an irreversible inactivation, but due to adsorption of the catalase to the glass-walls, setting in slowly when the hydrogen peroxide is nearly used up. At least within two half-periods the catalase is of practically constant activity and the first-order constant measures its concentration.

Table 1.

(Exp. 15 24/10, 52. $t = 20°$ C. $C_{Catalase} = 10^{-10}$ *M*. $C_{H_2O_2} \sim 10^{-3}$ *M*. pH = 7.0)
($C_{Na_2HPO_4} = 1/1500$ *M*; $C_{KH_2PO_4} = 1/1500$ *M*.)

t_{min}	ml Cerisol/g sol. = p $C_{H_2O_2}$	log p C	$\frac{\log C_0}{C}$	$\frac{1}{0.0625} \log \frac{C_0}{C} (= t)$
0	0.0518	0.7143−2	0	
1	0.0448	0.6513−2	0.0630	1.01
2	0.0388	0.5888−2	0.1255	2.01
4	0.0293	0.4669−2	0.2474	3.96
7	0.0191	0.2810−2	0.4333	6.95
	new H_2O_2 added at $t = 29$			
0	0.0592	0.7723−2	0	
1	0.0509	0.7076−2	0.0647	1.04
3	0.0387	0.5877−2	0.1846	2.96
5	0.0292	0.4654−2	0.3069	4.91
8	0.0186	0.2695−2	0.5028	8.05

$k' = 0.0625$

The catalase was prepared from horse kidney after Bonnichsens prescription [5]. It was kept in a *ca.* $2 \cdot 10^{-8}$ *M* solution with 0.01 *M* potassium chloride added to prevent adsorption to the glass walls (Svendsen [8]); the activity is then constant for some months. The catalase molarity was determined with a Beckman-Spectrophotometer, setting the extinction in millimolar solution at 405 mμ and 1 cm column to 380.

In Table 1 $k' = 0.0625$ is the constant at 20° C using decadic logarithms and the minute as time-unit and with a catalase molarity of 10^{-10}. Therefore:

$$k = \frac{0.0625}{0.4343} \cdot \frac{1}{60} \cdot 10^{10} = 2.40 \cdot 10^7 \text{ l} \cdot \text{moles}^{-1} \cdot \text{sec}^{-1}$$

This value is smaller than Svendsen's value: $2.65 \cdot 10^7$ (also for horse kidney catalase) and much less than that of Bonnichsen: $3.5 \cdot 10^7$ (for horse erythrocyte catalase). This is probably due to impurities in the catalase preparation, which was not perfectly crystalline.

The influence of azide on the first-order rate constant k' was studied in the following way, using Svendsen's [7] technique. Buffer-solution (1/1500 *M* Na_2HPO_4 + 1/1500 *M* KH_2PO_4, pH ~ 6.95), peroxide and azide are mixed in a black-painted reaction flask; at the time t_0 catalase is added from a micropipette, the flask is shaken, put into the thermostat at 20° C and the stopwatch is started. At intervals a sample is withdrawn, blown into a weighed flask containing enough 5 *M* sulphuric acid to make the solution about 0.5 *M* and weighed. Hydrogen peroxide is then titrated with 0.02 *M* ceric sulphate using "Ferroin" as indicator.

The konstant k' was determined with azide-concentrations varying from 10^{-8} to $1.2 \cdot 10^{-6}$ M and with pH $\sim$ 6.95. Plotting C_{azide} against $1/k'$ one gets a straight line which indicates the soundness in assuming a mass-action law behavior. Setting

$$\frac{C_{\text{active catalase}} \cdot C_{azide}}{C_{\text{inactive catalase}}} = K$$

and

$$\frac{C_{\text{active catalase}}}{C_{\text{inactive catalase}}} = \frac{k'}{k_0' - k'}$$

we get

$$C_{azide} = \frac{K k_0'}{k'} - K$$

$k_0' = 0.0645$ was found by extrapolation to $C_{azide} = 0$, consistent with the value found in Table 1 (0.0625) and other experiments without azide. $K = 0.92 \cdot 10^{-7}$ M was found from the slope of the line ($= K \cdot k_0'$).

Table 2. Comparison between the experimental values of k' for different azide concentrations and values calculated from

$$k'_{calc.} = \frac{0.92 \cdot 10^{-7} \; 0.0645}{C_{azide} + 0.92 \cdot 10^{-7}}$$

(pH = 6.95 ± 0.05; T = 20° C).

Azide conc.'s in moles/liter	k'_{exp}	$k'_{calc.}$	% deviation
12.05 10^{-7}	0.00448	0.00457	+ 2.0
10 —	0.00521	0.00543	+ 4.1
9 —	0.00604	0.00598	— 1.0
8 —	0.00641	0.00665	+ 3.6
6 —	0.00843	0.00857	+ 1.6
5 —	0.0103	0.01001	— 3.0
4 —	0.0131	0.01206	— 8.6
2 —	0.0211	0.0203	— 4.0
1 —	0.0299	0.0309	+ 3.2
0.5 —	0.0405	0.0418	+ 3.1
0.1 —	0.0614	0.0582	— 5.5
0	(0.0645) extrapol.		

Table 2 shows that a simple equilibrium is established between catalase and azide (at azide concentrations below $1.2 \cdot 10^{-6}$ M) with a mass action constant $K = 0.92 \cdot 10^{-7}$ moles/l at pH = 7 *. The interesting point is that one

* From a recent paper of Chance [9] one extrapolates the value $K \sim 10^{-3}$ (pH = 7), apparently for the same constant. The great difference between the two values seems to indicate, that our K is the mass-action constant of an equilibrium between azide plus a complex of catalase with hydrogen peroxide on one side and a complex with both hydrogen peroxide and azide on the other, whereas no hydrogen peroxide enters the equilibrium measured by Chance.

Our constant depends also on pH; the correct form for the equilibrium may therefore be:
active hydrogen peroxide catalase + N_3^- ⇌ inactive complex + $^{\cdot}OH^-$
The C_{OH^-} (at constant pH = 7) is incorporated in K.

single azide-ion is capable of inactivating the whole catalase molecule with its four hemin groups; these four groups must therefore be considered as a unity in spite of the fact, that the catalatic activity against hydrogen peroxide is not depending upon the existence of four groups; in horse-liver catalase there are only three and in spinach catalase one or two [10].

Acknowledgements: My sincere thanks are due to dr. E. Güntelberg and to dr. A. Svendsen for valuable information and help during the course of the work.

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Steady State. Treatment of Kinetc Results Concerning the Catalatic Decomposition of Hydrogen Peroxide

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To account for experimental results concerning the reaction in question a flow sheet is proposed which also implies an explanation of its retardation by azide ion. The essence is that azide acts as a competitive inhibitor by forming, reversibly, a stable complex with catalase containing one azide and at least one hydrogen peroxide.

It is known that at low hydrogen peroxide concentrations its catalatic decomposition up till nearly 100 % decomposition obeys the chronometric integral

$$kt = \ln\, h/x$$

where x is the concentration of hydrogen peroxide at time t, h the same at time zero and k the usual rate constant. The simplest reaction pattern which agrees with this result is

$$\begin{array}{ll} X_1 + H_2O_2 \rightleftharpoons X_2 & \pm 1 \\ X_2 + H_2O_2 \rightarrow 2H_2O + O_2 + X_1 & 2 \end{array}$$

X_1 may be either free catalase or catalase combined with one or several molecules of hydrogen-peroxide. Kinetics alone is unable to distinguish between these possibilities. X_2 obviously contains one molecule peroxide more than X_1.

Assuming steady state the usual procedure yields

$$\begin{aligned} x_1/s &= 1/w_1(1 + w_{-1}/w_2) \\ x_2/s &= 1/w_2 \end{aligned}$$

If furthermore the enzyme is either in the state X_1 or in X_2 we have $x_1 + x_2 = E$, where E is the total concentration of enzyme.

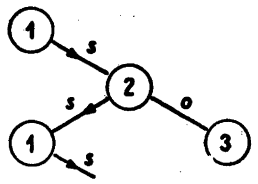

Fig. 1. The diagram is a flow sheet showing the transitions

$$X_1 + H_2O_2 \rightarrow X_2$$
$$X_2 + H_2O_2 \rightarrow 2H_2O + O_2 + X_1$$

and the equilibrium

$$X_2 + N_3^- \rightleftharpoons X_3.$$

The displacement of the two containers for X_1 indicates that there is a decrease in free energy by the overall reaction.

From the reaction pattern we conclude $w_1 = k_1x$, $w_{-1} = k_{-1}$; $w_2 = k_2x$, where x is the peroxide concentration. Insertion yields

$$-E\frac{dt}{dx} = (1 + k_{-1}/k_2x)/k_1x + 1/k_2x = E/s$$

This agrees only with the experimentally determined chronometric integral for small concentrations of peroxide if, for all accessible values of x, $k_{-1}/k_2x \ll 1$ and we thus get for the rate constant k : $1/k = 1_1/k_1 + 1/k_2$ which is in complete agreement with Beers and Sizer's [1] results.

Mrs. Skovsted's results concerning retardation by azide can be adequately interpreted by means of the flow-sheet (Fig. 1). We get as before

$$(x_1 + x_2)/s = 1/w_1 + 1/w_2.$$

But in this case $E = x_1 + x_2 + x_3$. x_3 is determined by the consideration that to preserve steady state the rate s_{23} must be zero, that is X_2 and X_3 are in equilibrium according to the chemical equation.

$$X_2 + A = X_3$$

which immediately yields $x_3 = Kax_2$. x_2 and x_3 are the concentrations of X_2 and X_3 respectively, while a is the concentration of azide. a may be considered as a constant if it is essentially larger than E. As $x_2/s = 1/w_2$ we thus get

$$(x_1 + x_2 + x_3)/s = E/s = 1/w_1 + (1 + Ka)/w_2$$

or

$$-x\,E\frac{dt}{dx} = 1/k_1 + (1 + Ka)/k_2 = 1/k'$$

where $k'E = k$ is the experimentally determined rate-constant. The expression obviously agrees completely with Mrs. Skovsted's results. The slope of $1/k'$ against a yields the numerical value of K/k_2. At present we have no means to determine k_1 and k_2 separately and it is therefore impossible to determine the numerical value of K. The result may also be written as follows

$$1/k = (1/k_1 + 1/k_2)/E + aK/k_2E$$
$$= (1/k_1 + 1/k_2)\,(1 + aKk_1/(k_1 + k_2))/E$$

Mrs. Skovsted's results may be written

$$1/k = (1 + 1.08 \cdot 10^7\ a)/0.0645 \cdot 2.303$$

Comparison yields

$$1/k_1 + 1/k_2 = E/0.0645 \cdot 2.303 \quad \text{where } E \sim 10^{-10} \text{ mole/liter}$$

and

$$Kk_1/(k_1 + k_2) = 1.08 \cdot 10^7.$$

Assuming that k_1 is essentially larger than k_2 we get

$$k_2 = 2.303 \cdot 0.0645/E$$

and

$$K = 1.08 \cdot 10^7$$

The large value of K indicates that X_2 which is catalase combined with at least one molecule peroxide has a very strong affinity for azide-ion.

It may also have a strong affinity for peroxide or peroxide anion, but while the complex with azide-ion is stable, the peroxide complex obviously explodes immediately after its formation.

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For other references see the preceding paper by Lis Skovsted.

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Über die Umsetzung von Formaldehyd mit Ketocarbonsäuren

II. Lävulinsäure *

Synthese des 3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lactons **

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Bei der erschöpfenden Oxymethylierung der Lävulinsäure in saurem Medium entsteht über die intermediär gebildete 3,3,5,5,5-Pentamethylol-lävulinsäure durch Wasserabspaltung das 3,5,5-Trimethylol-dihydro-desoxy-patulinsäurelacton, das aus dem Reaktionsgemisch als Methylenäther direkt isolierbar ist. Der Methylenäther lässt sich durch Alkali bzw. Formalin-Alkali zu einem Gemisch isomerer Methylenäther des 3,5,5-Trimethylol-tetrahydro-desoxy-patulinsäure-lactons reduzieren.

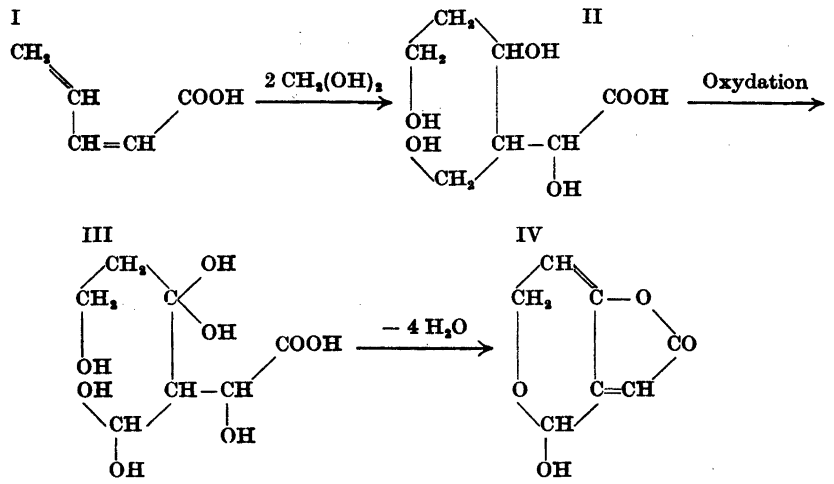
Unsere bisherigen Untersuchungen über die Umsetzung des Formaldehyds mit Carbonylverbindungen in saurem Medium haben zu dem Ergebnis geführt, dass bei Ketonen [1] und der Brenztraubensäure [2] im Anschluss an den vollständigen Methylolaufbau durch überschüssigen Formaldehyd eine Reduktion der Carbonylgruppe zur Carbinolgruppe nicht erfolgt. Dieser bezüglich der Ketocarbonylgruppe beobachtete und durch das saure Milieu bedingte Abschirmungseffekt trat auch bei neueren Untersuchungen an Ketocarbonylverbindungen [3] ausnahmslos in Erscheinung, so dass an dessen Allgemeingültigkeit nicht mehr zu zweifeln ist. Dieser Effekt ermöglicht die mühelose Synthese von Systemen mit einer β-Ketonalkohol-Gruppierung, die nach Tollens nur schwer fixierbar sind.

In der vorliegenden Untersuchung sollten diese Verhältnisse an der Lävulinsäure studiert werden, die aus einem ganz besonderen Grunde unser Interesse beanspruchte. In Verbindung mit Arbeiten in der Patulinreihe haben wir früher die Frage behandelt, ob sich mit Hilfe einer Formaldehyd-Olefin-Reaktion an der *Vinyl-acrylsäure* (I) die Synthese des Antibiotikums *Patulin* (IV) verwirklichen liesse. Theoretisch ergäbe sich die Aufbaumöglichkeit über

* I. Brenztraubensäure *Acta Chem. Scand.* **8** (1954) 47.

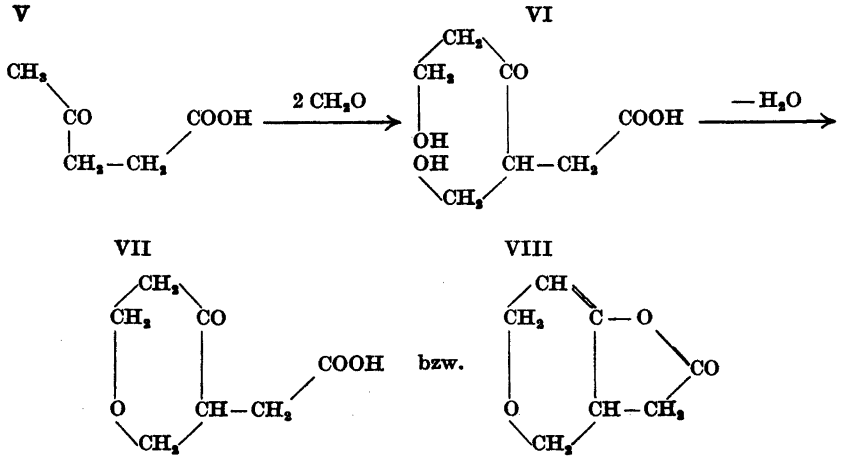
** 3. Mitt. über synthetische Versuche in der Reihe des Patulins und verwandter Verbindungen.

die Zwischenstufen II und III, die in ihrer verblüffenden Einfachheit die Frage aufdrängte, ob nicht möglicherweise die Natur diesen oder einen ähnlichen Weg beschreitet [4]:

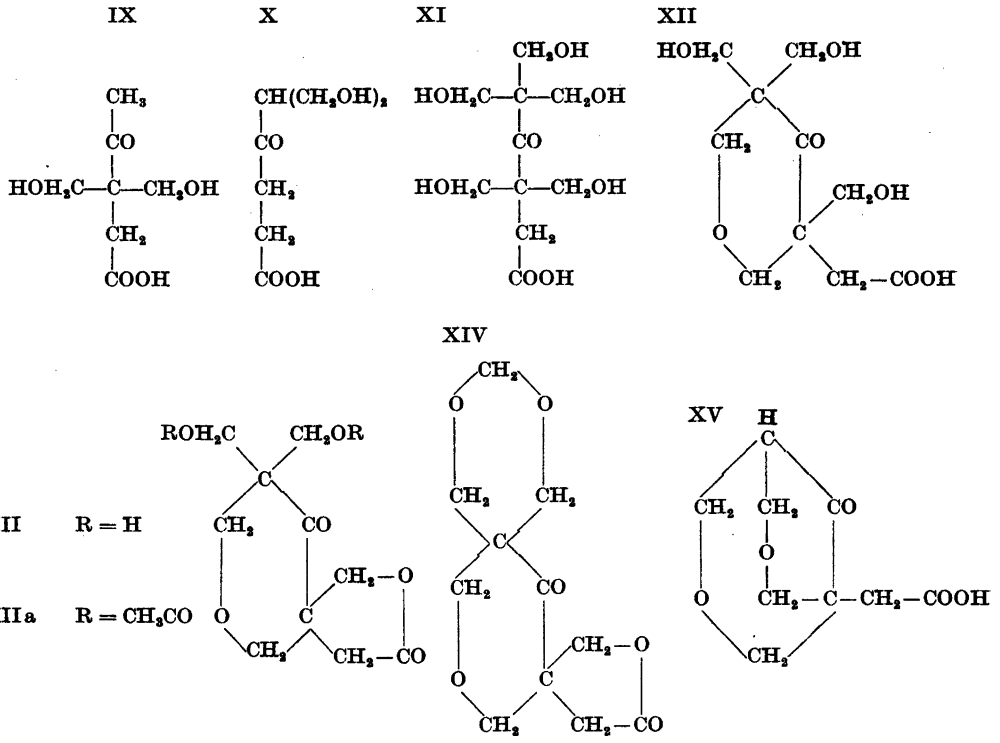


Obwohl man mit Hilfe der Formaldehyd-Olefin-Reaktion auch die Bildung einer Reihe anderer Naturstoffe erklären könnte, haben wir derartige Erwägungen aus naheliegenden Gründen vorläufig ganz in den Hintergrund gestellt.

Umsomehr überraschte es uns, als wir auf Grund der speziellen Reaktionsweise von Carbonylverbindungen das mögliche Resultat des Formaldehyd-Aufbaues an der *Lävulinsäure* (V) erwogen. Auch in diesem Falle führte unsere Überlegung über eine ebenso einfache Reaktionsfolge (V → VI → VII bzw. VIII) wieder in die Reihe des Patulins, nämlich zur *Dihydro-desoxy-patulinsäure* (VII) bzw. deren *Lactol* (VIII), wenn man annimmt, dass zwei Moleküle Formaldehyd reagieren:



Ein solcher Reaktionsverlauf hat zur Voraussetzung, dass sich die beiden Methylolgruppen am β- und δ-Kohlenstoffatom der Lävulinsäure verankern, während man natürlich mit der Möglichkeit rechnen muss, dass zuerst zwei Methylolgruppen am β- oder δ-Kohlenstoffatom ausgebaut werden, wodurch sie dann an einen für die Bildung der *Dihydro-desoxy-patulinsäure* falschen Platz gerieten (IX, X):



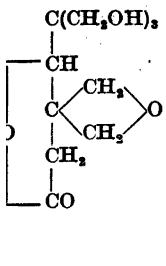
Bei Anwendung eines Formaldehyd-Überschusses (mehr als 5 Mol Formaldehyd) durfte man dagegen *mit Sicherheit* eine erschöpfende Oxymethylierung zur *Pentamethylol-lävulinsäure* (XI) erwarten, die sich durch Wasserabspaltung theoretisch in verschiedener Weise stabilisieren kann. Abspaltung von *einem* Molekül Wasser unter Tetrahydro-γ-pyron-Ringschluss führt zur *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure* (XII). Für die Abspaltung eines *zweiten* Moleküls Wasser aus dieser Säure sind zwei Möglichkeiten zu erneuter Ringschlussbildung vorhanden, nämlich Bildung des *γ-Lactons* (XIII) oder — im Falle eines nochmaligen Tetrahydro-γ-pyron-Ringschlusses — der *Säure* (XV).

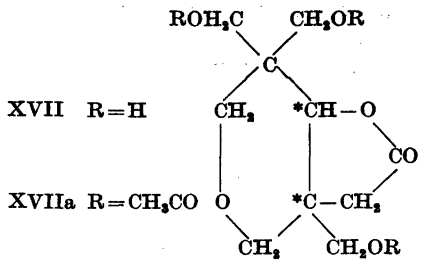
In der vorliegenden Studie haben wir uns hauptsächlich um die Synthese und die Chemie des "Per"-Oxymethylierungsproduktes der Lävulinsäure, der *Trimethylol-dihydro-desoxypatulinsäure* (XII) bzw. deren stabile "*Anhydroverbindungen*" (XIII bzw. XV) bemüht und daher die genaue Untersuchung

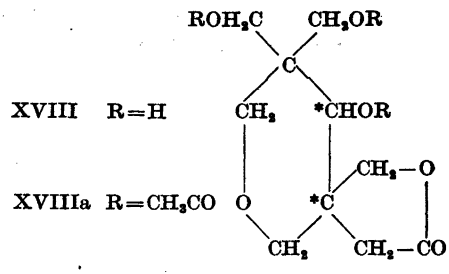
der Reaktions-Zwischenstufen — unter denen möglicherweise auch die *Dihydro-desoxy-patulinsäure* vertreten ist — einem späteren Zeitpunkte vorbehalten. Immerhin haben wir zur Orientierung Lävulinsäure und Formaldehyd auch in den Molverhältnissen 1 : 1, 1 : 2 und 1 : 3 umgesetzt und die *Dihydro-desoxy-patulinsäure* neben anderen Reaktionsprodukten nach Überführung in Methylestergemische durch fraktionierte Kristallisation der 2,4-Dinitro-phenylhydrazone vergeblich nachzuweisen versucht. Dieser Nachweis gelang auch nicht nach der Destillation der Methylestergemische, was vielleicht auf die Thermolabilität bestimmter Syntheseprodukte zurückzuführen ist, die auch bei geringen Drucken leicht Formaldehyd und Wasser abspalten unter Bildung ungesättigter Abbauprodukte. Im experimentellen Teil wird ein entsprechender Versuch beschrieben. Bei der Destillation erhielten wir neben einer Reihe anderer, bisher nicht näher untersuchter Substanzen ein farbloses Öl vom Sdp.$_9$ 153° von ungesättigtem Charakter. Die Analyse stimmt einigermassen auf die Summenformel $C_6H_8O_3$. Es handelt sich wahrscheinlich um die β- oder die *δ-Methylenlävulinsäure*, obwohl es merkwürdig ist, dass unter den angewendeten Bedingungen nicht der Methylester entstand. Aus diesem Grunde haben wir das isomere *β-Acetylbutyrolacton*, das als Reaktionsprodukt durchaus möglich wäre, in Erwägung gezogen. Tatsächlich gibt die Substanz mit Brady's Reagenz ein *2,4-Dinitrophenylhydrazon* $C_{12}H_{12}O_6N_4$ vom Schmp. 189—190°, was gegen das Vorliegen einer Methylenlävulinsäure spricht, da diese unter Brady's Bedingungen hätte methyliert werden müssen unter Bildung des *Methylester-2,4-dinitro-phenylhydrazons* $C_{13}H_{14}O_6N_4$. Andererseits konnte man aus der Substanz $C_6H_8O_3$ mit Hydrazinhydrat ein Derivat $C_6H_8ON_2$ vom Schmp. 225—226° gewinnen, für das nur die Formel des *Pyridazinon-Derivates* einer der beiden *Methylenlävulinsäuren* offensteht. Das ausserdem dargestellte *Semicarbazon* $C_7H_{11}O_3N_3$ vom Schmp. 194—195° lässt auch keine eindeutige Entscheidung zwischen den in Rede stehenden Isomeren zu. — Aus dem gleichen Versuch konnten wir ein weiteres 2,4-Dinitrophenylhydrazon $C_{13}H_{15}O_7N_4$ vom Schmp. 165—166° isolieren, das sich wahrscheinlich vom *Methylester der β-Methylol- oder δ-Methylol-lävulinsäure* ableitet.

Nach diesen orientierenden Versuchen über partielle Oxymethylierung haben wir uns ganz der erschöpfenden Oxymethylierung der Lävulinsäure zugewendet. Bei Anwendung eines Formaldehyd-Überschusses durften wir damit rechnen, die *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure* bzw. deren *Anhydroderivat* aus dem Reaktionsgemisch direkt als *Methylenäther* abscheiden zu können, von dem aus wir nach Reduktion und Methylenätherspaltung den Anschluss an eine frühere Arbeit von Rave und Tollens [5] herzustellen hofften. Diese hatten nämlich bei der alkalischen Kondensation der Lävulinsäure mit überschüssigem Formaldehyd eine Verbindung $C_{10}H_{16}O_6$ vom Schmp. 174—176° erhalten und hierfür die Formel eines *Anhydro-pentamethylol-hydroxy-valeriansäure-lactons* (XVI) aufgestellt, die wegen des Widerspruches zwischen dem darin angenommenen Trimethylenoxydring und der Überführbarkeit der Verbindung in ein *Triacetat* nicht richtig sein kann und durch eine der anschliessend näher zu begründenden Formeln eines *Trimethylol-tetrahydro-desoxy-patulinsäure-lactons* (XVII bzw. XVIII) ersetzt werden muss.

XVI







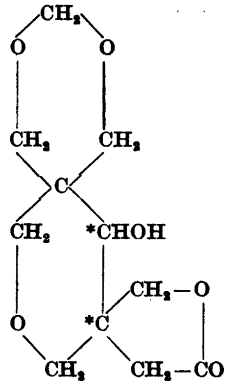
Bei der Umsetzung der Lävulinsäure mit Formaldehyd im Molverhältnis 1 : 7 bis 1 : 10 in Eisessig-Schwefelsäure erhielten wir aus dem Reaktionsgemisch ohne weiteres eine schön kristallisierende Verbindung $C_{11}H_{14}O_6$ vom Schmp. 162—163° in guter Ausbeute, die sich durch die folgenden Reaktionen als der *Methylenäther des 3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lactons* (kurz "Ketomethylenäther") (XIV) erwies. Dieser "Ketomethylenäther" reagiert in der Kälte nicht mit salzsaurem 2,4-Dinitro-phenylhydrazin. Beim Erwärmen mit diesem Reagenz in Gegenwart überschüssiger Salzsäure wird Formaldehyd unter Bildung des *Formaldehyd-2,4-dinitro-phenylhydrazons* abgespalten. Ausser durch Salzsäure gelingt die Spaltung der Methylenäther-Bindung durch Destillation mit methylalkoholischer Schwefelsäure nach Price und Krishnamurti [6]. Die besten Erfahrungen haben wir jedoch bei der Destillation mit Eisessig-Schwefelsäure gemacht, bei der der als Methylenäther gebundene Formaldehyd quantitativ abgespalten wird unter Entstehung eines bisher nicht rein dargestellten *Acetates* (XIII a), das sich mit methylalkoholischer Salzsäure leicht zum *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton* (XIII) vom Schmp. 129—132° umestern liess. Die Richtigkeit der Formel XIII und die Ausschliessung der Säureformel XV ergibt sich aus den Eigenschaften der Verbindung. Sie ist in Wasser leicht löslich. Die Carbonylgruppe lässt sich darin — wie vom 2,2,6,6-Tetramethylol-cyclohexanon her bekannt [7] — mit Carbonylreagenzien nicht nachweisen. Die Verbindung setzt aus Bicarbonatlösung kein Kohlendioxyd in Freiheit und reagiert in methanolischer Lösung nicht mit Diazomethan. Eine schwach alkalische Thymolphthaleinlösung wird nur langsam entfärbt. Nach Zusatz überschüssiger Lauge lässt sich die Substanz gegen Phenolphthalein scharf titrieren. Bemerkenswerterweise werden durch sie Tollenssche und Fehlingsche Lösung reduziert. Wir werden auf die Chemie dieser Verbindung in Kürze in einer gesonderten Abhandlung zurückkommen.

Der "*Ketomethylenäther*" (XIV) besitzt die Eigenschaft, beim Erhitzen mit wässrigem Alkali zum Teil in ein Gemisch isomerer *reduzierter Methylenäther* (kurz "Oxymethylenäther") überzugehen. Aus diesem Gemisch haben wir vorerst zwei isomere "Verbindungen" * $C_{11}H_{16}O_6$ mit den Schmelzpunkten

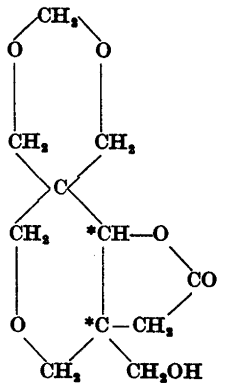
* Es ist möglich, dass die hier als "Verbindungen" bezeichneten "Oxymethylenäther" keine völlig einheitlichen Individuen darstellen, sondern isomerenverunreinigt sind, worauf das Verhalten beim Umkristallisieren und beobachtete Schmelzpunktsanomalien hindeuten. Unter diesem Vorbehalt sprechen wir im folgenden die "Oxymethylenäther" der angegebenen Schmelzpunkte als Verbindungen an.

184—187° bzw. 205—206° herauspräpariert, die sich vom *3,5,5-Trimethyloltetrahydro-desoxy-patulinsäure-lacton* (XVII bzw. XVIII) ableiten und denen daher eine der Formeln XIX oder XX zukommt.

XIX



XX



Die Reduktion des "Ketomethylenäthers" kommt offenbar dadurch zustande, dass dieser durch Alkali teilweise unter Abspaltung von Formaldehyd zerlegt wird, der dann — wie bei der Schlussphase der Tollensschen Reaktion — die Carbonylgruppe zur Carbinolgruppe reduziert. Bei diesem Reaktionsverlauf sollte man unter den Reaktionsprodukten auch das *3,5,5-Trimethyloltetrahydro-desoxy-patulinsäure-lacton* (XVII bzw. XVIII) erwarten, dessen Nachweis uns an dieser Stelle jedoch bisher, wahrscheinlich infolge der komplexen Natur der Gemische, noch nicht gelang. Bei der Reduktion des "Ketomethylenäthers" entsteht ein neues asymmetrisches Kohlenstoffatom, weshalb man ausser den durch die Formeln XIX und XX gekennzeichneten Strukturisomeren mit dem Vorliegen der entsprechenden Stereoisomeren rechnen muss. Diese Verhältnisse machen es zunächst unmöglich, den beiden isolierten "Oxymethylenäthern" eine bestimmte Formel zuzuschreiben, da hier strukturelle und sterische Fragestellungen ineinander greifen. Der niedriger schmelzende "Oxymethylenäther" vom Schmp. 184—187° liess sich durch Erhitzen mit Formalin-Salzsäure in das höher schmelzende Isomere vom Schmp. 205—206° umlagern. Die gleichen "Oxymethylenäther" erhielten wir in etwas besserer Ausbeute bei Versuchen, den "Ketomethylenäther" mit Formalin-Natronlauge bzw. Formalin-Kalilauge zu reduzieren. Im Gegensatz hierzu liess sich der "Ketomethylenäther" nicht mit Formalin-Salzsäure reduzieren. Die Möglichkeit zur Reduktion erschöpfend oxymethylierter Carbonylverbindungen durch Formalin-Alkali ergibt sich als eine logische Konsequenz der Tollensschen Reaktionsbedingungen. Während man aber beim Tollensschen Aufbau von Ketonen mit überschüssigem Formaldehyd in alkalischem Milieu die Reduktion der Carbonylgruppe nicht verhindern kann, hat man es nun in der Hand, durch saure Kondensation das "Per"-Oxymethylierungsprodukt als Methylenäther abzufangen und durch Milieuwechsel (sauer → alkalisch), falls erwünscht, den Reduktionshub getrennt zu besorgen.

In gleicher Weise wie den "Ketomethylenäther" haben wir versucht, die daraus erhaltenen "*Oxymethylenäther*" (XIX bzw. XX) an den Methylen-

ätherbrücken zu spalten, um so möglicherweise zu dem von Rave und Tollens beschriebenen Isomeren $C_{10}H_{16}O_6$ vom Schmp. 174—176° zu gelangen, obwohl wir — u.a. wegen der leichten Umlagerungsmöglichkeit des Lactonringsystems — auf die Entstehung eines schwer zu trennenden Isomerengemisches gefasst sein mussten. Merkwürdigerweise haben Rave und Tollens von derartigen Schwierigkeiten nichts erwähnt. Bei der Spaltung unseres höher schmelzenden "Oxymethylenäthers" vom Schmp. 205—206° mit Salzsäure-Aluminiumchlorid erhielten wir eine Substanz $C_{10}H_{16}O_6$ vom Schmp. 154—156°, für deren Einheitlichkeit wir uns aber aus den angeführten Erwägungen einstweilen nicht verbürgen können. Bei der Behandlung eines uneinheitlichen Präparates des "Oxymethylenäthers" mit Eisessig-Schwefelsäure enstand ein *Triacetat* $C_{16}H_{22}O_9$ (XVII a bzw. XVIII a) von unscharfem Schmp. 120—125°, während Rave und Tollens für das von ihnen dargestellte Triacetat einen Schmp. 161° angeben. Eine genauere Untersuchung der bei den "Oxymethylenäthern" vorliegenden Isomerieverhältnisse ist in Aussicht genommen. Aus den gleichen Gründen, wie für die "Oxymethylenäther" angeführt, können wir bezüglich der von uns dargestellten Substanz $C_{10}H_{16}O_6$ zwischen den für das *3,5,5-Trimethylol-tetrahydro-desoxy-patulinsäure-lacton* zur Wahl stehenden Formeln XVII und XVIII noch keine Entscheidung treffen. Wir nehmen jedoch als sicher an, dass sowohl die Verbindung von Rave und Tollens als auch unsere eigene Substanz Vertreter der durch diese Formeln beschriebenen Isomeren sind.

EXPERIMENTELLER TEIL

Unter Mitarbeit von Gustav Havre

I. Umsetzung der Lävulinsäure mit Formaldehyd (Molverh. 1:3)

Eine Mischung von 107 g Lävulinsäure (dargestellt nach Mentzer und Billet [8]), 85 g Paraformaldehyd, 250 ml Eisessig und 10 ml konz. Schwefelsäure wurde allmählich unter Rückflusskühlung erhitzt. Nach dem Abklingen der bei etwa 100° einsetzenden exothermen Reaktion wurde 3 Stunden gekocht. In der dann portweinroten Lösung liess sich nach dem Erkalten mit Fuchsinschwefliger Säure kein freier Formaldehyd mehr nachweisen. Zwecks Spaltung von Methylenäthern wurde die Flüssigkeit an der Brücke destilliert unter gleichzeitigem Zutropfen von Eisessig, wobei das Flüssigkeitsvolumen im Kolben konstant gehalten wurde. Nachdem 500 ml überdestilliert waren, wobei die Destillationstemperatur von 104° auf 118° anstieg, liess sich im Destillat mit salzsaurem 2,4-Dinitro-phenylhydrazin kein Formaldehyd mehr nachweisen. Das Reaktionsgemisch wurde mit 500 ml Wasser versetzt und mehrmals ausgeäthert. (Wässrige Flüssigkeit *A*.) Die Ätherauszüge wurden vereinigt, mit gesättigter Natriumsulfatlösung gewaschen und über wasserfreiem Natriumsulfat getrocknet. Die Ätherlösung, die noch viel Essigsäure enthielt, wurde vom Äther befreit und nach Zusatz von methylalkoholischer Salzsäure zwecks Veresterung der Essigsäure und Umesterung von Acetaten eine Zeitlang unter Rückflusskühlung gekocht. Anschliessend destillierte man unter Zutropfen von absolutem Methanol, bis beim Sdp. 65° nur noch reines Methanol überging. Zur Vervollständigung der Veresterungsreaktion behandelte man die eingedampfte Methanollösung mit überschüssigem Diazomethan. Bei der Destillation erhielt man als Hauptmenge (18 g) eine Fraktion von hellgelber Farbe und vom Sdp$_9$ 153°. Die Substanz, die in Wasser leicht löslich ist, entfärbt eine Brom-Chloroformlösung und gibt eine positive Baeyer-Reaktion.

β-Methylen-lävulinsäure (?) bzw. *δ-Methylen-lävulinsäure* (?) bzw. *β-Acetyl-butyrolacton* (?). (Gef. C 55,78, H 6,39. Ber. für $C_6H_8O_3$ (128,1): C 56,24, H 6, 29.)

2,4-Dinitro-phenylhydrazon: Eine Probe der Substanz wurde in Methanol mit Brady's Reagenz versetzt. Hellgelbe Kristalle aus Methanol. Schmp. 189—190°. (Gef. C 46,77; H 3,95; N 18,40. Ber. für $C_{13}H_{12}O_6N_4$ (308,3): C 46,75; H 3,93; N 18,18.)

Pyridazinonderivat: Beim 2-stündigen Erwärmen der Substanz mit Hydrazin-hydrat (85%) in methylalkoholischer Lösung auf dem Wasserbade erhielt man farblose Kristalle, die aus Methanol umkristallisiert bei 225—226° schmolzen. (Gef. C 58,09; H 6,55; N 22,75. Ber. für $C_6H_8ON_2$ (124,1): C 58,05; H 6,49; N 22,57.)

Semicarbazon: Farblose Blättchen aus Methanol. Schmp. 194—195° (zers.) (Gef. C 45,47; H 6,03. Ber. für $C_7H_{11}O_2N_3$ (185,2): C 45,39; H 5,99.)

Die *wässrige Flüssigkeit A* wurde zwecks Neutralisation der Schwefelsäure mit der berechneten Menge gesättigter Sodalösung versetzt, wobei sich ein braunes Öl am Boden abschied, das abgetrennt und nicht berücksichtigt wurde. Die nunmehr hellgelbe Flüssigkeit wurde mit Salzsäure kongosauer gemacht und auf dem Wasserbade eingedampft. Nach Verreiben des nicht ganz trockenen Eindampfrückstandes mit etwas wasserfreiem Natriumsulfat extrahierte man im Soxhletapparat 4 Stunden mit Äther, wobei sich am Boden ein zähflüssiges Öl abschied, das von der Ätherlösung durch Dekantieren getrennt wurde. Eine Probe des Öles wurde mit Brady's Reagenz versetzt. Der entstehende rotgelbe Niederschlag liess sich durch fraktionierte Kristallisation in eine Reihe verschiedener Substanzen zerlegen. Vorläufig wurde nur eine davon in reiner Form isoliert; die aus Methanol erhaltenen orangegelben Kristalle schmelzen bei 165—166° (Nach dem Wiedererstarren hat die Substanz den Schmp. 175°!). *2,4-Dinitrophenylhydrazon des Methylesters der β-Methylol- oder δ-Methylol-lävulinsäure* (?). (Gef. C 46,42; H 4,42; N 16,05. Ber. für $C_{13}H_{16}O_7N_4$ (340,3): C 45,88; H 4,74; N 16,47.)

II. Umsetzung der Lävulinsäure mit Formaldehyd (Molverh. 1 : 7,5)

Eine Mischung von 100 g Paraformaldehyd, 180 ml Eisessig, 13 ml konz. Schwefelsäure und 50 g Lävulinsäure wurde wie unter I. beschrieben zur Reaktion gebracht. Das hellbraune Reaktionsgemisch kristallisierte beim Stehen in der Kälte. Beim Absaugen erhielt man 43 g farbloser Kristalle, aus der Mutterlauge eine weitere Menge. Die Substanz, die durch Paraformaldehyd verunreinigt ist, kristallisierte man zuerst aus Wasser, dann aus Alkohol um. Schmp. 162—163°. V.Z. 243,0 (ber. 231,6). Die Verbindung spaltet beim Kochen mit Salzsäure Formaldehyd ab.

Methylenäther des 3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lactons (XIV), "*Ketomethylenäther*". (Gef. C 54,50; H 5,78. Ber. für $C_{11}H_{14}O_6$ (242,2): C 54,54; H 5,83.)

III. Spaltung des "Ketomethylenäthers" zum 3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton (XIII)

a) *mit Salzsäure-Aluminiumchlorid.* Eine Mischung von 5 g "Ketomethylenäther" und 5 g Aluminiumchlorid wurde vorsichtig mit 50 ml ca. 6 *N* Salzsäure versetzt. Die erhaltene Lösung wurde sodann unter gleichzeitigem Zutropfen von 6 *N* Salzsäure destilliert, bis sich im Destillat mit 2,4-Dinitro-phenylhydrazin-Hydrochlorid kein Formaldehyd mehr nachweisen liess. Die Flüssigkeit dampfte man im Vakuum weitgehend ein und verrieb den noch etwas feuchten Eindampfrückstand mit wasserfreiem Natriumsulfat zu einem trockenen Pulver, das im Soxhletapparat 12 Stunden mit abs. Äther extrahiert wurde. Man erhielt aus dem Ätherextrakt 4 g Rohprodukt, das aus Essigester umkristallisiert wurde (2,5 g). Schmp. 129—132°. Beim Erhitzen über den Schmelzpunkt spaltet die Verbindung langsam Formaldehyd ab. Sie reagiert in der Kälte nicht mit salzsaurem 2,4-Dinitrophenylhydrazin, reduziert Tollenssche und Fehlingsche Lösung und lässt sich in methylalkoholischer Lösung nicht mit Diazomethan methylieren. (Gef. C 52,38; H 6,14. Ber. für $C_{10}H_{14}O_6$ (230,2): C 52,17; H 6,13.)

b) *mit methylalkoholischer Schwefelsäure.* Die gleiche Verbindung wurde auch bei der Spaltung mit methylalkoholischer Schwefelsäure erhalten (Zuerst Kochen am Rückflusskühler, dann Abdestillieren des gebildeten Methylals unter gleichzeitigem Zutropfen von Methanol).

c) *mit Eisessig-Schwefelsäure* 450 g des rohen "Ketomethylenäthers" (verunreinigt durch Paraformaldehyd und Spuren Schwefelsäure) wurden mit 1 200 ml Eisessig und 5 ml

konz. Schwefelsäure * an der Brücke destilliert unter gleichzeitigem Zutropfen von reinem Eisessig, so dass das Flüssigkeitsvolumen im Kolben während der 3,5 Stunden dauernden Destillation annähernd konstant blieb (Wichtig!). Nach dieser Zeit war im Destillat kein Formaldehyd mehr mit 2,4-Dinitro-phenylhydrazin-Salzsäure nachweisbar und die Destillationstemperatur auf 118° gestiegen. Die im Kolben befindliche hellrote Flüssigkeit, die neben dem Diacetat (XIII a) überschüssige Essigsäure enthielt, wurde zwecks Veresterung der Essigsäure und gleichzeitiger Umesterung des Diacetates mit absolutem Methanol eine Zeitlang unter Rückflusskühlung gekocht und anschliessend unter Zutropfen von absolutem Methanol destilliert, bis die Siedetemperatur durch Abdestillieren des Methylacetates den Siedepunkt des reinen Methanols erreicht hatte. Aus der etwas eingeengten Lösung kristallisierten in der Kälte 330 g Rohprodukt, das durch Umkristallisieren aus Äthylacetat rein erhalten wurde. Auf diese Weise ist es leicht möglich, das 3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton in Ausbeuten von 70 % und darüber (bezogen auf Lävulinsäure!) zu erhalten.

IV. Verhalten des "Ketomethylenäthers" (XIV) gegenüber Alkali

Eine Mischung von 10 g des "Ketomethylenäthers", 15 g Kaliumhydroxyd und 50 ml Wasser wurde 2 Stunden gekocht. Das gelbe Reaktionsgemisch wurde nach dem Ansäuern mit Salzsäure (kongosauer!) ausgeäthert. Aus der über Natriumsulfat getrockneten Ätherlösung schied sich beim Eindampfen eine kristalline Substanz ab, die abgesaugt (1,8 g) (Mutterlauge *A*) und aus Methanol umkristallisiert wurde. Schmp. 205—206°. Die Verbindung reagiert nicht mit 2,4-Dinitro-phenylhydrazin-Salzsäure. V.Z. 255,5 (ber. 229,5). *Methylenäther des 3,5,5-Trimethyloltetrahydro-desoxy-patulinsäure-lactons* (XIX bzw. XX) (*"höherschmelzende"* Form). (Gef. C 53,98; H 6,60. Ber. für $C_{11}H_{16}O_6$ (244,2): C 54,09; H 6,60.)

Aus der *Mutterlauge A* kristallisierte nach einiger Zeit eine farblose Substanz (0,6 g). Schmp. 184—187° (aus Methanol). *Methylenäther des 3,5,5-Trimethylol-tetrahydro-desoxy-patulinsäure-lactons* (XIX bzw. XX) (*"niedrigerschmelzende" Form*). (Gef. C 53,98; H 6,69. Ber. für $C_{11}H_{16}O_6$ (244,2): C 54,09; H 6,60.)

Die gleichen Substanzen wurden bei der Behandlung des "Ketomethylenäthers" mit Natronlauge erhalten.

V. Reduktion des "Ketomethylenäthers" (XIV) mit Formalin-Natronlauge bzw. Kalilauge

Man löste 20 g des "Ketomethylenäthers" und 40 g Natriumhydroxyd in 400 ml Wasser, fügte 40 ml Formalinlösung (45 %) hinzu und erwärmte das Gemisch 7 Stunden auf dem Wasserbade. Die gelbe Flüssigkeit wurde mit konz. Salzsäure kongosauer gemacht und 5 mal mit je 200 ml Äther ausgeschüttelt. (Wässrige Schicht *A*). Beim Eindampfen der über Natriumsulfat getrockneten Ätherlösung schieden sich Kristalle aus, die abgesaugt und aus Methanol umkristallisiert wurden. Schmp. 184—187°. Mischprobe mit dem unter IV. beschriebenen "Oxymethylenäther" $C_{11}H_{16}O_6$ ("niedrigerschmelzende" Form) unverändert. — Beim Stehen der *wässrigen Flüssigkeit A* schied sich eine farblose Substanz vom Schmp. 202—205° ab, die sich bei der Mischprobe mit der "höherschmelzenden" Form des "Oxymethylenäthers" $C_{11}H_{16}O_6$ identisch erwies. — Bei der Umsetzung des "Ketomethylenäthers" mit Formalin-Kalilauge erhielt man nach dem Eindampfen des angesäuerten Reaktionsgemisches zur Trockne und Extraktion im Soxhletapparat nur die *"niedrigerschmelzende"* Form $C_{11}H_{16}O_6$ vom Schmp. 184—186° in 25 % Ausbeute.

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* Enthält das Ausgangsmaterial Spuren Schwefelsäure, lassen sich an Stelle der Schwefelsäure mit Vorteil 10—20 ml konz. Salzsäure verwenden, wodurch man nach der Umesterung leichter zu einem völlig farblosen Präparat gelangt.

VI. Umlagerung der "niedrigerschmelzenden" Form des "Oxymethylenäthers (XIX bzw. XX) in die "höherschmelzende" Form

0,8 g des "Oxymethylenäthers" $C_{11}H_{16}O_6$ vom Schmp. 184—187° wurde in einer Mischung von 15 ml Formalin und 15 ml konz. Salzsäure kalt gelöst und die Lösung 3 Stunden auf dem Wasserbade erhitzt. Die nun gelbe Flüssigkeit dampfte man weitgehend ein. Nach 2 Tagen hatten sich Kristalle ausgeschieden, die abgesaugt und aus Methanol umkristallisiert wurden. Schmp. und Mischschmp. mit dem "höherschmelzenden" Isomeren $C_{11}H_{16}O_6$ unverändert 204—206°.

VII. Spaltung des "höherschmelzenden Oxymethylenäthers" (XIX bzw. XX) mit Salzsäure-Aluminiumchlorid

1 g des "Oxymethylenäthers" vom Schmp. 204—206° wurde, wie unter III a) für den "Ketomethylenäther" beschrieben, mit 1 g Aluminiumchlorid und 6 *N* Salzsäure behandelt und aufgearbeitet. Man gewann so 0,2 g farblose Kristalle vom Schmp. 154—156° (aus Essigester): *3,5,5-Trimethylol-tetrahydro-desoxy-patulinsäure-lacton* (XVII bzw. XVIII). (Gef. C 51,71; H 6,93. Ber. für $C_{10}H_{16}O_6$ (232,2): C 51,72; H 6,95.)

VIII. Spaltung des "Oxymethylenäthers" (XIX bzw. XX) mit Eisessig-Schwefelsäure

2 g "Oxymethylenäther" (Isomerengemisch; Schmp. ca. 170—195°) wurden mit 150 ml Eisessig und 2 ml konz. Schwefelsäure unter Zutropfen von reinem Eisessig destilliert. Nach 90 Minuten liess sich im Destillat mit 2,4-Dinitro-phenylhydrazin-Hydrochlorid kein Formaldehyd mehr nachweisen. Der Kolbeninhalt wurde nach Zusatz von 6 g wasserfreiem Natriumacetat im Vakuum zur Trockne eingedampft. Durch Extraktion des Eindampfrückstandes im Soxhletapparat mit abs. Äther erhielt man farblose Kristalle, die nach mehrfachem Umkristallisieren aus Eisessig unscharf zwischen 120—125° schmolzen.

Triacetat des 3,5,5-Trimethylol-tetrahydro-desoxy-patulinsäure-lactons (XVII a bzw. XVIII a). (Gef. C 53,82; H 6,29. Ber. für $C_{16}H_{22}O_9$ (358,3): C 53,63; H 6,19.)

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Note on the Preparation of Acetals of Alkoxysuccinaldehydes

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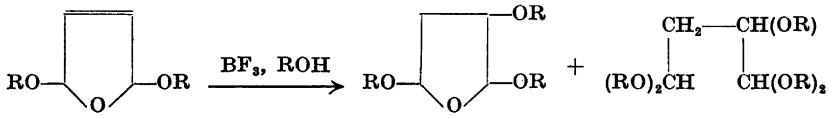
Acetals of alkoxysuccinaldehydes have been prepared by interaction of alcohols and 2,5-dialkoxy-2,5-dihydrofurans in the presence of catalytic amounts of boron fluoride etherate. The use of a new setup for laboratory fractional batch distillations is demonstrated.

Stoll, Lindenmann and Jucker [1] have prepared 2,3,5-trialkoxytetrahydrofurans by interaction of equimolar amounts of 2,5-dialkoxy-2,5-dihydrofurans and hydrogen bromide in alcoholic solution. These authors suggest that the reaction takes place through an intermediate 2,5-dialkoxy-3-bromotetrahydrofuran in which the bromine is replaced by a metathetic reaction with the solvent.

We have obtained trimethoxy- and triethoxytetrahydrofurans by interaction of the alcohol and the dihydrofuran in the presence of an acid catalyst, preferably boron fluoride etherate (*cf.*[2]). At the same time a certain amount of the tetraalkyl acetal of the alkoxysuccinaldehyde is formed



55 % (R = Et)
33 % (R = Me)

16 % (R = Et)
32 % (R = Me)

The reaction mixtures were analyzed by fractional distillation and, as expected (*cf.*[3]), it was found that the trialkoxytetrahydrofurans consisted of mixtures of two or more stereoisomers. It was also found that the reaction mixture from 2,5-dimethoxy-2,5-dihydrofuran contained 9 % of malealdehyde tetramethyl acetal.

We believe that the experiments of Stoll, Lindenmann and Jucker [1] are also acid-catalyzed alcohol additions rather than two-step reactions proceeding through an intermediate bromo compound.

The results of the distillations are given in detail in order to demonstrate the use of a new setup for laboratory fractional batch distillations [4].

Table 1. Distillation of 56.76 g of ethyl acetals under 14 mm; heat input 4.0 watt; take-off every 2.4 minutes; change of fraction every 1.0 hour; total yield of distillate 55.75 g (98.2 %).

Fraction (g)	B. p. °C	n_D^{25}	C %	H %	OC_2H_5 %
1 (1.10)	< 89	1.4243			
2 (1.82)	89 –100.1	1.4230			
3 (1.86)	100.1–100.4	1.4220	59.3	10.1	65.0
$C_4H_5O(OC_2H_5)_3$ (204.3) requires			58.8	9.9	66.2
4 (1.84)	100.4–100.6	1.4219			
5 (1.85)	100.6	1.4219			
6 (1.85)	100.6–100.7	1.4219			
7 (1.83)	100.7–100.8	1.4218			
8 (1.83)	100.8–100.9	1.4219			
9 (1.79)	100.9	1.4219	59.0	10.2	65.5
10 (1.79)	100.9	1.4219			
11 (1.85)	100.9–101.0	1.4219			
12 (1.79)	101.0	1.4220			
13 (1.82)	101.0–101.1	1.4220			
14 (1.76)	101.1–101.2	1.4220			
15 (1.79)	101.2–101.6	1.4222			
16 (1.83)	101.6–102.0	1.4227			
17 (1.75)	102.0–102.3	1.4229			
18 (1.81)	102.3–102.4	1.4231			
19 (1.79)	102.4–102.7	1.4231			
20 (1.82)	102.7–103.1	1.4231			
21 (1.85)	103.1–104.0	1.4231	59.4	10.1	65.0
22 (1.74)	104.0–120	1.4231			
23 (1.78)	120 –135.0	1.4202			
24 (1.74)	135.0–136.0	1.4171			
25 (1.70)	136.0	1.4167	60.4	10.8	80.3
$C_4H_5(OC_2H_5)_5$ (278.4) requires			60.4	10.9	80.9
26 (1.73)	136.0	1.4166			
27 (1.69)	136.0–136.1	1.4165			
28 (1.72)	136.1	1.4164	60.7	10.9	80.3
29 (1.65)	136.1	1.4164			
30 (1.69)	136.1–136.2	1.4164			
31 (1.68)	136.2	1.4163	61.3	11.3	80.4
32 (1.21)	136.2–135.0	1.4167			

EXPERIMENTAL

Microanalyses in part by K. Glens

Addition of ethanol to diethoxydihydrofuran. Diethoxydihydrofuran [5] (150.0 g), ethanol (anhydrous, 560 ml) and boron fluoride etherate (15.0 ml) were mixed and heated under reflux (90 min). A solution of sodium ethoxide [from sodium (7.5 g) and ethanol (60 ml)] was added and the mixture heated further under reflux (30 min). This was done in order to destroy traces of compounds with a free aldehyde group. After cooling, the borofluoride was removed by filtration and washed with ether. The ethanol and the ether were distilled in a vacuum from a water-bath (70°). The excess of sodium ethoxide was removed by centrifugation and washed twice with ether. The reaction product combined with the etheral washings was then distilled. The yield was 167.0 g of a colorless liquid, $b.p._{17}$ 101—135°. The product slowly forms peroxides on standing.

The product was fractionally distilled in a previously described setup including a 12 cm column [4]. The result of the distillation is shown in Table 1.

The yield of triethoxytetrahydrofuran (fractions 3—22) was 36.24 g (55 %) and of ethoxysuccinaldehyde tetraethyl acetal (fractions 24—32) 14.81 g (16 %). The yield of the mixture of acetals is 52.83 g (fractions 3—32) (74 %).

It is apparent from Table 1 that the triethoxytetrahydrofuran consists of a mixture of two or more stereoisomers. Stoll, Lindenmann and Jucker [1] report $b.p._{15}$ 91—98° for their triethoxytetrahydrofuran.

Addition of methanol to dimethoxydihydrofuran. A mixture of acetals of methoxysuccinaldehyde was prepared from dimethoxydihydrofuran [6] (100.0 g), methanol (dried with magnesium, 400 ml) and boron fluoride etherate (10.0 ml) as described above. The yield was 121.0 g of a colorless liquid, $b.p._{15}$ 75—102°. The product was distilled through a 25 cm column [4] (Table 2). During the distillation a low boiling product (0.58 g in all, n_D^{25} 1.3531), probably consisting mainly of methanol, accumulated in the cooling trap. In order to reduce this splitting off of methanol, the product rate was increased towards the end of the distillation.

In the article describing the setup for fractional distillation [4] it was claimed that when a pure compound is distilled at constant boil-up rate and a fixed setting of the timer, the product rate remains constant within 3 %. In the above distillations this is only true for the distillation shown in Table 1, but not for the distillation shown in Table 2. We believe that the reason for this is the very slight decomposition which takes place during the latter distillation. The formation of gas causes small variations of the pressure in the still-pot and the column, whereby the boil-up rate and thereby the product rate is changed. In our experience, a slight decomposition is actually indicated more clearly by variations of the product rate, rather than by variations of the boiling point or the purity of the product (*e. g.* as measured by the refractive index).

The yield of trimethoxytetrahydrofuran (fractions 2—12) was 40.79 g (33 %), of malealdehyde tetramethyl acetal (fractions 15—18) 12.28 g (9 %) and of methoxysuccinaldehyde tetramethyl acetal (fractions 20—32) 50.46 g (32 %). The yield of the mixture of acetals (fractions 2—32) is 113.76 g (81 %).

The malealdehyde tetramethyl acetal (previously found [7] $b.p._{760}$ 198—199°, n_D^{25} 1.4282) was identified by transformation into malealdehyde bis-phenylhydrazone as described previously [6]. The yield was 74 mg (72 %), m. p. 170—171° (Kofler stage, corr.). The hydrazone was further characterized by the infrared absorption spectrum (in potassium bromide), which was identical with that of an authentic specimen prepared from dimethoxydihydrofuran. (Found: C 72.3; H 6.2; N 21.5. Calc. for $C_{16}H_{16}N_4$ (264.3): C 72.7; H 6.1; N 21.2).

It is apparent from Table 2 that the trimethoxytetrahydrofuran is a mixture of two or more stereoisomers. Stoll, Lindenmann and Jucker [1] report $b.p._{15}$ 78—82° for their product. Kebrle and Karrer [8] report b $p._{13}$ 85—86° and n_D^{20} 1.4300 for a product prepared by methylation of a 2,5-dimethoxy-3-hydroxytetrahydrofuran.

Table 2. Distillation of 119.6 g of methyl acetals under 25 mm; heat input 3.6 watt; take-off every 6.9 minutes; change of fraction every 8 hours. After about half of fraction 26 was collected and until the end of the distillation the take-off valve was opened every 1.7 minutes and the fractions changed every 4 hours. The total yield of distillate, including the product in the cooling trap, was 116.9 g (97.7 %).

Fraction (g)	B.p. °C	n_D^{25}	C %	H %	OCH_3 %
1 (2.52)	65.5– 81.3	1.4296			49.0
2 (3.87)	81.3– 88.3	1.4233			54.7
3 (3.74)	88.3– 88.5	1.4228	52.0	8.9	55.8
$C_4H_5O(OCH_3)_3$ (162.2) requires			51.8	8.7	57.4
4 (3.65)	88.5– 88.6	1.4223			55.3
5 (3.72)	88.6	1.4225			56.3
6 (3.67)	88.6– 88.9	1.4222			57.7
7 (3.65)	88.9– 89.0	1.4226	51.7	9.0	56.4
8 (3.57)	89.0– 89.2	1.4226			56.7
9 (4.03)	89.2– 90.1	1.4230			56.4
10 (3.73)	90.1– 92.3	1.4241			55.9
11 (3.68)	92.3– 93.5	1.4260	51.3	8.8	56.4
12 (3.48)	93.5– 95.2	1.4264			57.0
13 (3.41)	95.2–101.3	1.4252			59.5
14 (3.18)	101.3–103.2	1.4233			65.3
15 (3.10)	103.2–104.3	1.4242			67.0
16 (3.05)	104.3–104.8	1.4252			68.5
17 (3.04)	104.8–105.1	1.4259	54.5	9.2	69.0
$C_4H_4(OCH_3)_4$ (176.2) requires			54.5	9.2	70.4
18 (3.09)	105.1–111.5	1.4261			69.0
19 (3.64)	111.5–119.1	1.4191			71.5
20 (3.48)	119.1–119.2	1.4169	52.1	10.1	73.0
$C_4H_5(OCH_3)_5$ (208.3) requires			51.9	9.7	74.5
21 (2.94)	119.2	1.4169			
22 (3.01)	119.2	1.4168			
23 (3.10)	119.2	1.4168	52.4	9.9	74.5
24 (2.95)	119.2	1.4167			
25 (3.07)	119.2	1.4167			
26 (4.39)	119.2	1.4167			
27 (5.47)	119.2	1.4166	52.4	9.8	74.0
28 (5.51)	119.2	1.4167			
29 (5.84)	119.2	1.4166			
30 (5.92)	119.2	1.4168			
31 (3.90)	119.2	1.4167	51.8	9.7	73.2
32 (0.88)	119.2	1.4176			72.8

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Studies of Absorption Spectra

VIII. Three and More d-Electrons in Cubic Crystal Fields

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The first order splitting of d^n-terms in relatively weak crystal fields of cubic symmetry is obtained by application of the diagonal sum rule to the intermediate crystal field case, first studied by Bethe. In some of the cases, where the same crystal field quantum number Γ_n applies to two or more levels of the same multiplicity, the method can only give the average value of the term splitting. Terms of d^5, which are only represented once, cannot show first order splitting in the cubic field.

Bethe [1] found the number of levels, originating from a term with a given L by group-theoretical means for any type of crystal field symmetry. In the simplest case, the cubic symmetry, the terms of two d-electrons were investigated by Ilse and Hartmann [2]. The present paper gives some of the analogous results for d^3, d^4 and d^5-systems. The splitting of d^{10-n}-terms is inverted, respectively to the corresponding d^n-terms. When two or more terms with the same combination of L and S occur, or when two levels of the same term have the same [1] crystal field quantum number Γ_n, only the average value of the splitting of the combined levels can be found by this method. As shown below, the splitting of D- and H-terms with the same S cannot be found separately, because of a similar conformity.

In strong crystal fields the levels can be classified [1] according to their number of γ_3- and γ_5-electrons, while they have no fixed L as the terms in the free ion. Table 1 gives the distribution of the levels for the different electron numbers. The cubic crystal field quantun numbers Γ_n can be found by factorizing the group products of γ_3 and γ_5 of the occurring electrons * as linear combinations of the character system of Γ_n, given in Table 1, Ref[1]. The multiplicity $2S + 1$ of the levels can be found by consideration of the degene-

* When two or more electrons with the same γ_n are present, their group product must be restricted by Pauli's exclusion principle. This can be done by comparison with the number of terms in the free ion, split by a cubic crystal field, or by comparison with the number of totally non-degenerate states in fields of lower (tetragonal or rhombic) symmetry.

racy number $(2S + 1)\,e$, where $e = 1, 1, 2, 3, 3$ for Γ_n with $n = 1, 2, 3, 4, 5$, respectively. The total sum of degeneracy numbers is given in the last column of Table 1.

The following relation between d^{10-n}-levels and d^n-levels can be used for higher numbers of d-electrons:

$$\gamma_5^a \gamma_3^b \equiv \gamma_5^{6-a} \gamma_3^{4-b} \tag{1}$$

Special cases of this analogy to Pauli's equivalence theorem are $\gamma_5^a \equiv \gamma_5^{6-a}$ and $\gamma_3^b \equiv \gamma_3^{4-b}$.

If E_1 is the energy of a γ_3-electron and E_2 of a γ_5-electron, the energy of the d^n-levels can be written:

$$E = E(\text{free ion}) + NE_1 + (n-N)E_2 \tag{2}$$

It is often convenient to fix the zero-point of the energy scale by defining the energy $\frac{2n}{5} E_1 + \frac{3n}{5} E_2$ which is constant for all levels of the n electrons to be $= 0$, and thus to write:

$$E = E(\text{free ion}) + (N - \frac{2n}{5})(E_1 - E_2) \tag{3}$$

The second part of the expression gives the deviation of the level from the centre of gravity of the term, defined according to Bethe [1]. The Orgel diagram [3] is E of equ. 3 as function of (E_1-E_2). This parameter indicates the relative strength of the crystal field and is the energy difference between the two states of one d-electron in the same cubic field. In some cases, the value of N changes from a limiting value for small (E_1-E_2) to another value for large (E_1-E_2), as pointed out by Santen and Wieringen [4]. The latter limiting values can be found in Table 1 as the number of γ_3-electrons.

However, in the cases, where only one level ${}^{2S+1}\Gamma_n$ occurs in a given electron number in Table 1, N is invariable for all values of (E_1-E_2). Thus, the d^2-levels have $N = 1$ in ${}^1\Gamma_4$ and ${}^3\Gamma_5$, corresponding[2] to the first-order values $E_1 + E_2$ for ${}^1G$ and ${}^3F$ and $N = 2$ in ${}^3\Gamma_2$ from ${}^3F$. For d^3-systems is $N = 0$ in ${}^4\Gamma_2({}^4F)$ and $N = 1$ in the three cases ${}^2\Gamma_1({}^2G)$, ${}^2\Gamma_2({}^2F)$ and ${}^4\Gamma_5({}^4F)$. For d^4-systems is $N = 1$ for ${}^3\Gamma_1({}^3G)$. As also found by Santen and Wieringen[3] is $N = 1$ for ${}^5\Gamma_3({}^5D)$ and $N = 2$ for ${}^5\Gamma_5({}^5D)$.

In the cases where two or more ${}^{2S+1}\Gamma_n$ are represented, the total number of γ_3-electrons occurring in the levels must equal the total sum of N for any value of (E_1-E_2). This is analogous to the diagonal sum rule of Condon and Shortley [5]. *E.g.*, in d^2-systems, the two ${}^3\Gamma_4$ have together [2] $N = \frac{4}{5} + \frac{1}{5} = 1$ and the two ${}^1\Gamma_3$ have together $N = \frac{8}{7} + \frac{6}{7} = 2$.

The distribution of the N-sum on the individual levels for $(E_1-E_2) \to 0$ can be found from the theorems of Bethe [1] on the deviations from the centre of gravity of the original term. For S- and P-terms, which do not split up in a cubic field, N is invariably $\frac{2n}{5}$. For D-terms the energy can be expressed:

$$E(\Gamma_3) = E(D) + 3\eta(D) \text{ and } E(\Gamma_5) = E(D) - 2\eta(D) \tag{4}$$

Table 1. Distribution of d^n levels in strong crystal fields of cubic symmetry.

Electron configuration:		Total sum of degeneracy numbers
d: γ_3	$^2\Gamma_3$	4
γ_5	$^2\Gamma_5$	6
d^2: γ_3^2	$^1\Gamma_1 + {}^3\Gamma_2 + {}^1\Gamma_3$	6
$\gamma_5\gamma_3$	$^1\Gamma_4 + {}^3\Gamma_4 + {}^1\Gamma_5 + {}^3\Gamma_5$	24
γ_5^2	$^1\Gamma_1 + {}^1\Gamma_3 + {}^3\Gamma_4 + {}^1\Gamma_5$	15
d^3: γ_3^3	$^2\Gamma_3$	4
$\gamma_5\gamma_3^2$	$2\,{}^2\Gamma_4 + {}^4\Gamma_4 + 2\,{}^2\Gamma_5$	36
$\gamma_5^2\gamma_3$	$^2\Gamma_1 + {}^2\Gamma_2 + 2\,{}^2\Gamma_3 + 2\,{}^2\Gamma_4 + {}^4\Gamma_4 + 2\,{}^2\Gamma_5 + {}^4\Gamma_5$	60
γ_5^3	$^4\Gamma_3 + {}^2\Gamma_3 + {}^2\Gamma_4 + {}^2\Gamma_5$	20
d^4: γ_3^4	$^1\Gamma_1$	1
$\gamma_5\gamma_3^3$	$^1\Gamma_4 + {}^3\Gamma_4 + {}^1\Gamma_5 + {}^3\Gamma_5$	24
$\gamma_5^2\gamma_3^2$	$2\,{}^1\Gamma_1 + {}^1\Gamma_2 + {}^3\Gamma_2 + 3\,{}^1\Gamma_3 + {}^3\Gamma_3 + {}^1\Gamma_4 + 3\,{}^3\Gamma_4 + 3\,{}^1\Gamma_5 + 2\,{}^3\Gamma_5 + {}^5\Gamma_5$	90
$\gamma_5^3\gamma_3$	$^1\Gamma_1 + {}^3\Gamma_1 + {}^1\Gamma_2 + {}^3\Gamma_2 + {}^1\Gamma_3 + 2\,{}^3\Gamma_3 + {}^5\Gamma_3 + 2\,{}^1\Gamma_4 + 2\,{}^3\Gamma_4 + 2\,{}^1\Gamma_5 + 2\,{}^3\Gamma_5$	80
γ_5^4	$^1\Gamma_1 + {}^1\Gamma_3 + {}^3\Gamma_4 + {}^1\Gamma_5$	15
d^5: $\gamma_5\gamma_3^4$	$^2\Gamma_5$	6
$\gamma_5^2\gamma_3^3$	$^2\Gamma_1 + {}^2\Gamma_3 + 2\,{}^2\Gamma_3 + 2\,{}^2\Gamma_4 + {}^4\Gamma_4 + 2\,{}^2\Gamma_5 + {}^4\Gamma_5$	60
$\gamma_5^3\gamma_3^2$	$2\,{}^2\Gamma_1 + {}^4\Gamma_1 + {}^6\Gamma_1 + {}^2\Gamma_2 + {}^4\Gamma_2 + 3\,{}^2\Gamma_3 + 2\,{}^4\Gamma_3 + 4\,{}^2\Gamma_4 + {}^4\Gamma_4 + 4\,{}^2\Gamma_5 + {}^4\Gamma_5$	120
$\gamma_5^4\gamma_3$	$^2\Gamma_1 + {}^2\Gamma_3 + 2\,{}^2\Gamma_3 + 2\,{}^2\Gamma_4 + {}^4\Gamma_4 + 2\,{}^2\Gamma_5 + {}^4\Gamma_5$	60
γ_5^5	$^2\Gamma_5$	6

The parameter $\eta(D)$ describes thus the splitting of the D-term and $E(D) = E(D \text{ free ion}) + \frac{2n}{5}E_1 + \frac{3n}{5}E_2$. According to Bethe [1], it is further possible to write for F-terms and G-terms:

$$\begin{aligned} E(\Gamma_2) &= E(E) + 6\eta(F) & \qquad E(\Gamma_1) &= E(G) + 14\eta(G) \\ E(\Gamma_4) &= E(F) - 3\eta(F) & \qquad E(\Gamma_3) &= E(G) + 2\eta(G) \\ E(\Gamma_5) &= E(F) + \eta(F) & \qquad E(\Gamma_4) &= E(G) + 7\eta(G) \\ & & \qquad E(\Gamma_5) &= E(G) - 13\eta(G) \end{aligned} \tag{5}$$

The parameters η can be compared with the Lande interval factor ζ for multiplet splitting[5] due to coupling between L and S.

When equ. 5 is applied to the case of $^2\Gamma_4$ in d^3-systems (or $^3\Gamma_4$ in d^4-systems) by use of the known values of $\eta(F)$ and $\eta(G)$, it can be concluded that the splitting of H-terms is of the form

$$\text{average of two} \quad \begin{aligned} E(\Gamma_3) &= E(H) + 3\eta(H) \\ E(\Gamma_4) &= E(H) \\ E(\Gamma_5) &= E(H) - 2\eta(H) \end{aligned} \tag{6}$$

Since similar results are not known for I-terms, $S = 0$ has not yet been treated in d^4-systems by the present method.

Table 2. Multiplet splitting factors $\eta(L)$ of systems with two, three and four d-electrons, expressed in units of (E_1-E_2).*

d^2	S	d^3	S	d^4	S
$\eta(D)=+4/35$	0	$2\eta(D_{av})+\eta(H)=+8/105$	$^1/_2$	$\eta(D)=-1/5$	2
$\eta(F)=+1/5$	1	$\eta(F)=-1/5$	$^3/_2$	$\eta(D)+\eta(H)=-5/21$	1
$\eta(G)=+1/35$	0	$\eta(F)=-1/30$	$^1/_2$	$2\eta(F_{av})=-1/60$	1
		$\eta(G)=-1/70$	$^1/_2$	$\eta(G)=-3/70$	1

* Finkelstein and Van Vleck[11] have given the matrices for d^3 in cubic fields. $\eta(H)$ is separately $\frac{1}{15}(E_1-E_2)$ and η of the two ^2D-terms $\frac{1}{10}$ and $\frac{-19}{210}$, giving the sum in Table 2. The first-order deviations of the two $\Gamma_4(^2H)$ levels are given[11] as $\pm$ 0.261 (E_1-E_2) which is an energy of the same order of magnitude as the non-diagonal elements in the matrices.

Equ. 6 has the interesting consequence that only the sum $\eta(D)+\eta(H)$ can be determined by this method in cases where D- and H-terms have the same S, since $\eta(D)$ and $\eta(H)$ only occur in proportional equations. This is connected with the fact that the character systems can be written

$$C(H) = 2C(P) + C(D) \tag{7}$$

This is not a sufficient cause for the peculiar degeneracy of equ. 6. Thus the expression

$$C(G) = C(S) + C(P) + C(D) \tag{8}$$

has no significant effect of the energy expressions. Generally the character systems can be written as a linear combination with the coefficients $A_n \geq 0$ of only four (and not five) fundamental character systems:

$$C(L) = A_0C(S) + A_1C(P) + A_2C(D) + A_3C(F) \tag{9}$$

Thus, the number of Γ_5 levels at a given L is always equal to A_2+A_3, the sum of Γ_3 and Γ_2 levels.

The results of Table 2 are obtained from the diagonal sum arguments above and equations 4—6. The subscript "av" denotes the average energy of the terms in the cases where two terms with the same combination of L and S are found in the free ion [5].

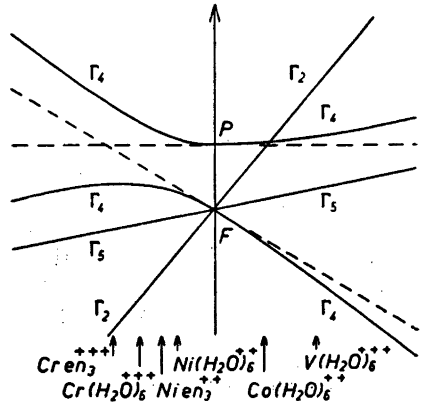

Fig. 1. Orgel diagram for terms of highest multiplicity in cubic crystal fields. Left-hand side d^3- and d^8-systems in octahedral complexes with negative ions or dipoles as ligands. Right-hand side d^3- and d^7-systems.

When the method is applied to d^5, all the $\eta(L)$ are 0. This could also be inferred from the inversion of the first order term splittings in d^{10-n} relative to d^n, since d^5 is its own hole-equivalent. The observed splittings [6] of ^4G in Mn^{++} and Fe^{+++} are thus due to second order effects of $(E_1—E_2)$ in stronger crystal fields (here acting on $^4\Gamma_4$ and $^4\Gamma_5$ for the two first bands). Since these terms usually have only small distances in the free ions with d^3, d^4 and d^5-configurations, their interaction can be described by the non-diagonal elements in the matrix of the form given by Orgel[6] which are presumably quite prominent for S smaller than the maximum value. E (free ion) of equ. 3 is also intermixed of the different interacting states, originating in the weak crystal field.

As an example of the transition from weak to strong crystal fields, Fig. 1 illustrates the interaction of levels with the same Γ_n in the case where the highest multiplicity is represented by F- and P-terms. The right-hand side is the Fig. 3 of Ref.[3], valid for d^2 and d^7, while the left-hand side applies to d^3 and d^8 in octahedral complexes [4]. Some concrete examples of crystal field strengths are given by the vertical arrows. The corresponding observed wave numbers are given in Table 3. It is seen that $\Gamma_5(F)$ is lower in d^3 and d^8-systems than the lowest of the two Γ_4. At high values of $(E_1—E_2)$, Γ_4 should in these systems be placed a constant amount below Γ_5 according to Orgel [3]. This is supported by the tetragonal splitting of the first band of mono- and trans-di-substituted chromium(III) complexes, since Γ_4 has first order tetragonal effects in contrast to Γ_5. Nevertheless, a crossing of Γ_4 and Γ_5 seems highly improbable. Rather the wave-functions are intermixed [1] by passing from weak to strong crystal fields. The third band in chromium(III) complexes, corresponding to the transition to the highest Γ_4 is predicted far in the ultra-violet.

Table 3. Maxima of observed absorption bands in cm^{-1}.

Shoulders are given in parenthesis. "en" = ethylenediamine. The energy difference between the P and F term of highest multiplicity is given for the free ion, known from atomic spectroscopy [10].

$V(H_2O)_6^{+++}$	17 300	25 800			$^3P—^3F$ = 13 300
$Cr(H_2O)_6^{+++}$	17 400	24 500			$^4P—^4F$ = 14 200
Cr en_3^{+++}	21 850	28 450			—»—
$Co(H_2O)_6^{++}$	8 000	(16 000)	19 400	(21 550)	$^4P—^4F$ = 15 400
$Ni(H_2O)_6^{++}$	8 000	13 500	(15 400)	25 300	$^3P—^3F$ = 16 900
Ni en_3^{++}	11 200	18 350	29 000		—»—

Analogous to the generalization from the d^2-levels to most of the d^n-levels in cubic crystal fields, Ballhausen and the author will publish elsewhere[12] results on d^n-levels in crystal fields of tetragonal and rhombic symmetry. These will utilize the calculations on one and two d-electrons in fields of low symmetry, according to Ballhausen [7,8,9], where the electron distributions in strong crystal fields on the individual tetragonal and rhombic quantum numbers γ_n can also be connected with the energy expressions in weak crystal fields of the corresponding symmetry for most d^n-systems.

Acknowledgment. I am very much indebted to Professor Jannik Bjerrum for his interest in this work, and to Mr. C. J. Ballhausen for many valuable discussions.

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The Crystal Structure of $(NH_4)_2SbCl_5$

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$(NH_4)_2SbCl_5$ has been investigated by X-ray methods. It is monoclinic, space group No. 12, $C2/m$-C_2^{3h} with 4 formula units in the unit cell. The cell dimensions are: $a = 11.9_8$ Å, $b = 7.7_6$ Å, $c = 11.9_7$ Å, $\alpha = 114.7°$ and $V = 1003.2$ Å^3, the atomic coordinates are given on p. 130, and the interatomic distances in Table 3. The antimony and chlorine positions were determined from Patterson projections and by applying the "Vector Convergence Method". The parameters were refined by electron density projections, from which the ammonium positions also were discovered. The ammonium positions were checked from space considerations.

The structure is represented in Figs. 11, 12, 13, and 14. Antimony is surrounded by five chlorine at five of the corners of a distorted octahedron forming an isolated $SbCl_5^{2-}$ group. The chlorine and ammonium form together a distorted close-packing with some vacant positions.

The structure determinations of the antimony(III)oxide halogenides $Sb_4O_5X_2$ where X = Cl or Br (Edstrand [1]), SbOCl (Edstrand [2]) and two other oxide halogenides with the idealized formulae $Sb_8O_{10}(OH)_2X_2$ where X = Cl, Br or I and $[Sb_8O_8(OH)_4]Cl_{2+x}[(OH)_{2-x}(H_2O)_{1+x}]$ (Edstrand[3]) have made it seem of interest to investigate the distances between trivalent antimony and the halogenide ions in the solid state, and also to obtain some more information regarding the coordination of trivalent antimony. In SbOCl there are two different types of Sb—Cl distances, one of the magnitude 2.4 Å and the other of the magnitude 3 Å. In $Sb_4O_5Cl_2$ we have only Sb—Cl distances of the magnitude 3 Å and larger. The distance of about 2.4 Å is of the same magnitude as the Sb—Cl distances found for $SbCl_3$ in the gas phase. Gregg, Hampson, Jenkins, Jones, and Sutton [4] found it to be 2.37 ± 0.02 kX by electron diffraction methods, and Kisliuk [5] found it to be 2.325 ± 0.005 Å by microwave methods.

Trivalent antimony seems to have different coordination numbers in different compounds. We shall here give a short review, which is not supposed to be complete, of the coordinations which have been found.

3-Coordination.

A more or less regular trigonal pyramid with antimony at the top: $SbCl_3$, $SbBr_3$, and SbI_3 in the gas phase [4,6], SbF_3 in the solid state [7], KSb_4F_{13} [8], Sb_2O_3 cubic [9,10] and orthorhombic [11], $ZnSb_2O_4$ [12], Tetrahedrite Cu_3SbS_3 [13,14], and Wolfsbergite $CuSbS_2$ [15,16].

In the following compounds there are Sb(III) atoms with different coordination numbers, one of which is three: $Sb_4O_5Cl_2$ and $Sb_4O_5Br_2$ [1], SbOCl [2], Sb_2S_3 [16,17] and the isotypic Sb_2Se_3 [18], and Berthierite $FeSb_2S_4$ [19].

4-Coordination.

I. Tetrahedral coordination: Berthierite $FeSb_2S_4$ [19].

II. A square pyramid with antimony at the top: Nadorite $PbSbO_2Cl$ [20].

III. A deformed trigonal bipyramid with antimony in the centre and with one of the equatorial corners unoccupied: $Sb_4O_5Cl_2$ and $Sb_4O_5Br_2$ [1], SbOCl [2], $Sb_8O_{10}(OH)_2X_2$ where X = Cl, Br, or I [3], and $[Sb_8O_8(OH)_4]Cl_{2+x}[(OH)_{2-x}(H_2O)_{1+x}]$ [3].

IV. A deformed trigonal bipyramid with antimony in the centre and with one of the tops unoccupied: $CsSb_2F_7$ [21].

5-Coordination.

A more or less regular octahedron with antimony in the centre and with one of the corners unoccupied: K_2SbF_5 [22], $KSbF_4$ [23], and $NaSbF_4$ [24]. In these papers there are also discussions of the different coordinations found in some complex antimony(III)fluorides. It seems as if half of the antimony atoms in Sb_2S_3 [16,17,25] have a coordination which can be interpreted as a 5-coordination of this type.

6-Coordination.

A more or less regular octahedron with trivalent antimony in the centre: $(NH_4)_2SbBr_6$, Rb_2SbCl_6, and Rb_2SbBr_6 [26], Sb_2O_4 and $SbTaO_4$ [27], and the idealized structure for Miargyrite $AgSbS_2$ [28].

8-Coordination.

Around the trivalent antimony in Sb_3O_6OH [29].

9-Coordination.

In SbSX [30] and the isotypic SbSeX [31] where X = Br or I. There is, however, close contact between Sb and only some of the 9 anions. To us the constitution of the double bands of Sb, S, and X seems to suggest 5-coordination.

In some of the examples the positions of the light atoms might be a little uncertain on account of the difficulty of locating them from the X-ray data and, accordingly, there might also be some uncertainty in the coordination number. In the cases of the coordination number 3, the results might have been influenced by a preconception that this should be the normal coordination of trivalent antimony.

For the investigation of antimony-halogen distances in the solid state it seemed more convenient to use the complex antimony(III)halogenides than the simple halogenides, as some of the former are more stable in air than the

latter. It also seemed best to start with a compound with a fairly simple formula and which did not contain any water of crystallization.

After the structure determination of $(NH_4)_2SbCl_5$ was finished, there appeared a structure determination of the complex chloride $Cs_3Sb_2Cl_9$ by Yamatera and Nakatsu[32]. They report that it has the same type of structure as $Cs_3As_2Cl_9$[33]. From the data by Yamatera and Nakatsu it does not seem as if the coordinations of As and Sb are the same in these compounds. In $Cs_3As_2Cl_9$ there is only a pseudocoordination of six around As as it is in close contact with only three Cl atoms. The data for $Cs_3Sb_2Cl_9$, however, seem to indicate six coordination for Sb, and when calculating the Sb—Cl distances we find them to be 2.7 Å, which seems rather large. The structure determination has been based on powder data, but is going to be re-examined by single crystal methods according to a private communication by Dr. K. Nakatsu.

PREPARATION

Complex antimony(III)halogenides have been prepared by many investigators. A number of complex compounds in the system NH_4Cl-$SbCl_3$ are described in Gmelin[34]. $(NH_4)_2SbCl_5$ was first prepared by Jacquelain[35] who obtained it from an acid solution of the two salts in mole proportions according to the formula and described it as a dodecahedron derived from a regular hexahedral prism. Dehérain[36] prepared it by treating $SbCl_3 \cdot 2NH_3$ with hydrochloric acid and described it as yellow hexagonal plates.

In the present investigation, $SbCl_3$ and NH_4Cl were mixed in the mole proportions 3:4 in water with or without hydrochloric acid. The solution was then evaporated by heat or by means of a vacuum. When a solution was evaporated by heating to the beginning of crystallization a lot of hexagonal leaves precipitated during the cooling and after that needle-shaped crystals were formed as four-sided prisms. An example is: 0.646 g NH_4Cl was dissolved in 2.5 ml of 1 *M* HCl and then mixed with 2.06 g $SbCl_3$. This solution was then evaporated by boiling to a volume of about 1 ml. A few hexagonal plates precipitated. It was then kept in a vacuum desiccator above sulfuric acid for 20 hours during which the main part crystallized as four sided prisms. As far as we know these four sided prisms have not been described in the literature before, unless they are identical with a compound found by Poggiale[37]. He described it as crystallizing in beautiful rectangular prisms and ascribed to it the formula $3NH_3$, HCl; $SbCl_3 + 1.5H_2O$. We used these crystals for the structure determination. They were fairly stable in air, but on long exposure to air they were hydrolysed.

ANALYSIS

Since we could not take any reliable powder photographs on account of the hygroscopic character of the substance, we had to pick out a single crystal from each preparation and take a rotation or Weissenberg photograph for the identification of the substance. To be sure that these crystals really represented the main part we proceeded in the following way. In one preparation we obtained a very large crystal. From this crystal we cut off a small fragment, of which we took a Weissenberg photograph. The rest of the crystal we used for antimony analysis, and found that it gave the same result as the other preparations.

For the antimony determination, the substance was dissolved in hydrochloric acid and titrated with potassium bromate according to Smith and May[38] with naphthol blue-black as indicator. For the determination of chlorine, the substance was boiled with a concentrated solution of sodium carbonate in a platinum dish. After filtration the chloride was titrated by the Volhard method in the presence of nitrobenzene. Ammonium was determined according to Kjeldahl.

	Ammonium %	Antimony %	Chlorine %
Calc. for $(NH_4)_2SbCl_5$	10.77	36.33	52.90
Found	10.37, 10.57	36.47, 36.17	52.13, 51.86

UNIT CELL AND SPACE GROUP

Single crystals were selected. To avoid their decomposition they were coated with Apiezon grease, which protected them fairly well. Rotation and Weissenberg photographs were taken round two of the axes with Cu-radiation. We had the following series of Weissenberg photographs (double films): *h*0*l*, *h*1*l*, *h*2*l*, *h*3*l*, *h*4*l*, *hk*0, *hk*1, *hk*2, *hk*3, *hk*4, *hk*5, *hk*6, and *hk*7. Relative intensities of the reflections were estimated visually by comparison with an intensity scale obtained by exposing an interval of the zero layer line, containing a strong reflection, with different exposure times on a pack of four double films. They were then corrected for the Lorentz and polarization factors using the curves given by Kaan and Cole [39].

The crystals proved to be monoclinic with the *b*-axis coinciding with the needle-axis. The cell dimensions were determined from the Weissenberg photographs, as we could not obtain reliable powder photographs. Using $\lambda_{CuK\alpha_1} = 1.54051$ Å, where the α_1 and α_2 reflections were separated, and $\lambda_{CuK\alpha} = \frac{1}{3}(2\lambda_{\alpha_1} + \lambda_{\alpha_2}) = 1.54176$ Å, where these reflections were not separated, we found the following values:

$a = 11.9_8$ Å, $b = 7.7_0$ Å, $c = 11.9_7$ Å, $\beta = 114.7°$ and $V = 1003.2$ Å^3

If 4 formula units are assumed per unit cell, the density would be $d_{calc} = 2.22$, we found $d_{obs} = 2.26$, 2.24.

The density of the crystals was determined from the loss of weight in carbon tetrachloride, as this liquid was found not to decompose the crystals. The density of carbon tetrachloride was taken from Beilstein [40].

In the Weissenberg photographs all reflections with $(h + k)$ odd are systematically absent, which is characteristic of the space groups No. 12, $C2/m$—C^3_{2h}, No. 5, $C2$—C^3_2, and No. 8 Cm—C^3_s [41]. To try to decide between these three space groups, we applied the method of intensity statistics [42, 43] to the zero zones *h*0*l* (see Fig. 1) and *hk*0 (see Fig. 2). It is apparent that the intensity distributions in both cases seem to indicate symmetry centres in

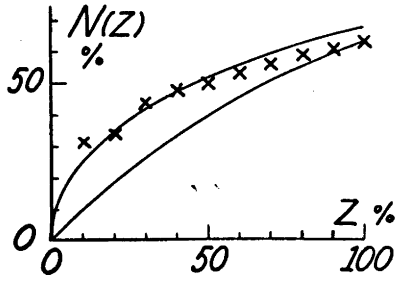

Fig. 1. Intensity statistics for h0l. × represents the experimental intensity distribution compared with the theoretical centric distribution (upper curve) and the theoretical acentric distribution (lower curve).

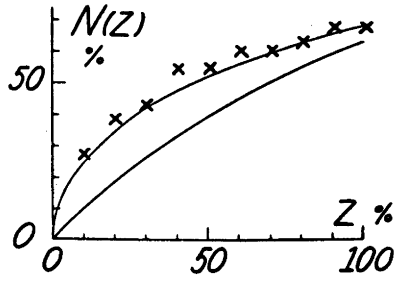

Fig. 2. Intensity statistics for hk0. × represents the experimental intensity distribution compared with the theoretical centric distribution (upper curve) and the theoretical acentric distribution (lower curve).

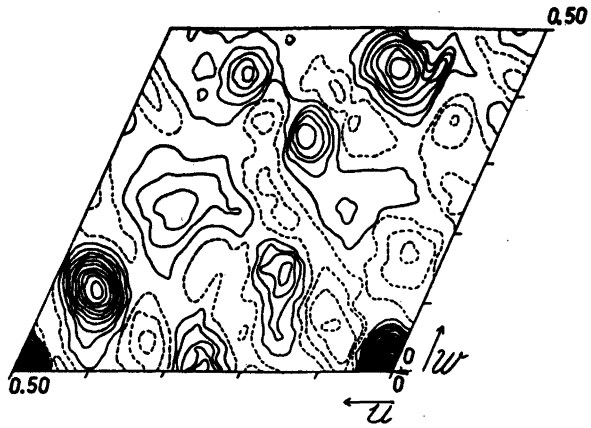

Fig. 3. P(UpW). Contours at an interval of 200 arbitrary units. Negative values dotted.

the projections, and thus it appeared reasonable to assume the space group to be No. 12, $C2/m$—C_{2h}^3, since this is the only one which has symmetry centres in both projections.

PATTERSON SYNTHESES AND VECTOR CONVERGENCE DIAGRAMS

It seemed probable that the positions of the four antimony atoms would be found from Patterson projections. The calculated $P(UpW)$-projection is given in Fig. 3 and the $P(UVp)$-projection in Fig. 4. Considering the space group $C2/m$, the antimony atoms can be situated either in one of the fourfold positions or in a combination of two twofold positions. Combinations of the twofold positions $2(a) + 2(c)$, $2(a) + 2(d)$, $2(b) + 2(c)$, and $2(b) + 2(d)$ all require maxima of the weight 2 in the $P(UpW)$-projection at $u = 0, w = \frac{1}{2}$ and at $u = \frac{1}{2}, w = \frac{1}{2}$. As we can see there are practically no observed maxima at these coordinates, and thus it seems reasonable to exclude the combinations mentioned. Combinations of the twofold positions $2(a) + 2(b)$ and $2(c) + 2(d)$ require maxima of the weight 2 in the $P(UVp)$-projection at $u = 0$, $v = \frac{1}{2}$. The observed maximum is, however, one of the lowest in this projection, and

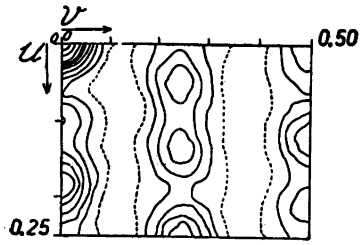

Fig. 4. P(UVp). Contours at an interval of 100 arbitrary units. Negative values dotted.

thus these combinations do not seem very probable. It therefore seems possible to exclude all the twofold positions. In the Patterson space $P(UVW)$ the fourfold positions give the following vectors:

I within 4(*e*) and 4(*f*) of weight 4	A	$0,\frac{1}{2},0$
	B	$\frac{1}{2},\frac{1}{2},0$
	C	$\frac{1}{2},0,0$
II within 4(*g*) and 4(*h*) of weight 4	A	$\frac{1}{2},\frac{1}{2},0$
of weight 2	B	$\pm(0,2y,0)$
	C	$\pm(\frac{1}{2},\frac{1}{2}+2y,0)$
III within 4(*i*) of weight 4	A	$\frac{1}{2},\frac{1}{2},0$
of weight 2	B	$\pm(2x,0,2z)$
	C	$\pm(\frac{1}{2}+2x,\frac{1}{2},2z)$

As the maximum in the $P(UVp)$-projection corresponding to the vector IA is among the lowest, it seems possible to exclude the positions 4(*e*) and 4(*f*). The only maximum corresponding to the vector IIB in $P(UVp)$, which is of reasonable height, is situated at the origin. As this corresponds to $y = 0$ or $\frac{1}{2}$, this would mean that the fourfold position corresponds to two twofold positions, which does not seem probable from what has been stated above.

We then have to consider only the position 4(*i*) and the corresponding vectors of group III, which ought to be among the highest maxima found in the projections. The vector IIIB corresponds to the following series of possible maxima in $P(UVp)$ with $u = 2x = 0$, 0.1025, 0.1847, 0.2908, 0.3818, 0.5000, 0.6182, 0.7092, 0.8153, 0.8975, 1.000 ..... where the maxima at $u = 0.1847$ and 0.8153 are the highest after that the origin. In $P(UpW)$ the two maxima next in height to that at the origin are at a) $u = 0.4403$, $w = 0.1198$, which corre-

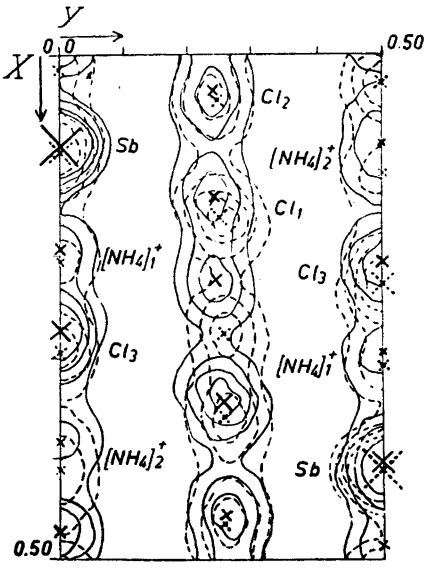

Fig. 5. The xy-vector convergence diagram. Only the lowest positive contours in P(UVp) have been drawn. The centers of the peaks are marked with crosses, the size of which are proportional to the heights. One set of Patterson maxima is marked with full lines and the other with dotted lines. The finally accepted atomic positions are indicated at the points of convergence.

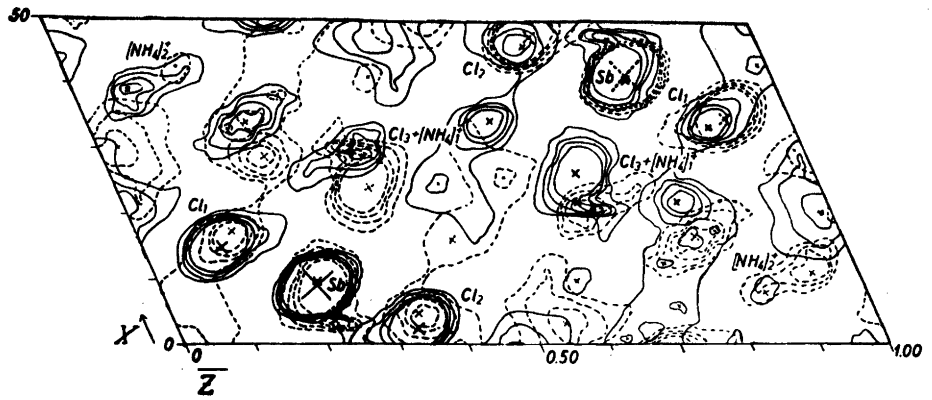

Fig. 6. The xz-vector convergence diagram. Only the lowest positive contours in P(UpW) have been drawn. The centers of the peaks are marked with crosses, the size of which are proportional to the heights. One set of Patterson maxima is marked with full lines and the other with dotted lines. The finally accepted atomic positions are indicated at the points of convergence.

sponds to the following series of u-values: 0.4403, 0.9403, 0.0597, and 0.5597 and b) $u = 0.1743$, $w = 0.4422$, which corresponds to the following series of u-values: 0.1743, 0.6743, 0.3257, and 0.8257. We can see that the u-values of the series a) and b) which are sufficiently close to values in the series from $P(UVp)$ are $u = 0.1743$ and 0.8257 (in $P(UVp)$ 0.1847 and 0.8153). These two u-values with corresponding maxima give four different sets of atomic coordinates, which, however, can be transformed into each other by translations of $\frac{1}{2}$ along the axes. We now choose the coordinates $x = \frac{1}{2}(\frac{1}{2}\ 0.1743 + \frac{1}{2}\ 0.1847) = 0.090$, $y = 0$, and $z = \frac{1}{2}\ 0.4422 = 0.221$ for Sb.

We also have to locate 20 chlorine atoms and 8 ammonium ions. It seemed quite probable that some of the other observed maxima in the Patterson projections would correspond to Sb—Cl vectors. This made it worth while to try the "Vector Convergence Method" described by Beevers and Robertson [44] on the projections starting with the previously found coordinates for Sb in 4(*i*). In this way we obtained the *xy*-projection in Fig. 5 and the *xz*-projection in Fig. 6. There are quite clear indications of the Cl-positions and it is also possible to guess something about the NH_4^+-positions. An interpretation of the diagrams gave the following set of approximate parameters, where the NH_4^+-positions are omitted since they did not seem quite certain.

		x	*y*	*z*
Sb	in 4(*i*)	0.090	0	0.221
Cl_1	in 8(*j*)	0.159	0.245	0.120
Cl_2	in 8(*j*)	0.048	0.243	0.350
Cl_3	in 4(*i*)	0.286	0	0.360

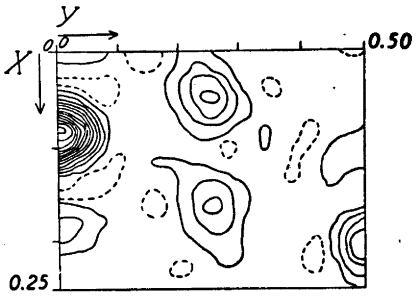

Fig. 7. ϱ (XYp). Contours at an interval of 100 arbitrary units. Negative values dotted.

A rough calculation of the interatomic distances with these preliminary parameters showed that the distances were of reasonable magnitude, and that Sb is surrounded by 5 Cl at five of the corners of a deformed octahedron. Since a coordination of this type has been reported previously for the SbF_5^{2-}-ion in K_2SbF_5 by Byström and Wilhelmi [22], the Cl-positions seemed quite likely. It can be mentioned that $(NH_4)_2SbCl_5$ and K_2SbF_5 are not isomorphous.

FOURIER SYNTHESES

The best way to refine the parameters of antimony and chlorine and try to find the ammonium positions was by Fourier syntheses. In this case the coordination around antimony is of much more interest than the coordination around the ammonium ions, and thus it does not seem necessary to find the ammonium parameters with very large accuracy. The Fourier syntheses have been calculated with $F_{obs} = \sqrt{\frac{I}{Lp}}$ where F is the structure factor, I is the estimated intensity, Lp are the combined Lorentz and polarization factors. In all syntheses the value of $F(000)$ has been omitted. Correction for absorption has not been applied. The maxima have been located by the interpolation table given by Booth [45].

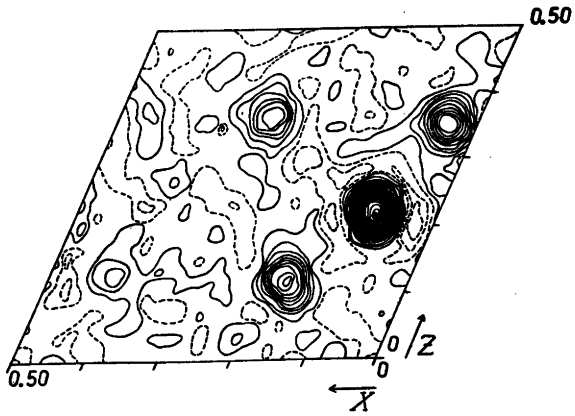

Fig. 8. ϱ (XpZ). Contours at an interval of 200 arbitrary units. Negative values dotted.

The refinement proceeded by successively calculating several $\varrho(XYp)$- and $\varrho(XpZ)$-projections, including more and more F-values. The final $\varrho\,(XYp)$-projection is given in Fig. 7, and the final $\varrho(XpZ)$-projection in Fig. 8. The antimony and chlorine peaks are clearly resolved, and we see that, in the $\varrho\,(XpZ)$-projection, the eightfold chlorine positions Cl_1 and Cl_2 are higher than the fourfold chlorine position Cl_3 as can be expected. There are also indications of the ammonium positions, which seem to be situated in the fourfold positions 4(*i*). One of them, $(NH_4^+)_1$, is quite clear in $\varrho\,(XYp)$ but is masked in $\varrho\,(XpZ)$ by Cl_3, since the two atoms are situated practically above each other. The $(NH_4^+)_2$-maximum in $\varrho\,(XpZ)$ is the maximum next in height to the Cl_3-peak. It is also found in ϱ (XYp) but here it is rather low. We then arrived at the following set of parameters, where x_1 indicates the parameter from $\varrho\,(XpZ)$, x_2 the parameter from $\varrho\,(XYp)$, x_{mv} the mean value of the two, and $\triangle x = |x_1 - x_2|$.

		x_1	x_2	x_{mv}	y	z	$\triangle x$
4 Sb	in 4(*i*)	0.0860	0.0867	0.086_3	0	0.220_8	0.0007 = 0.008 Å
8 Cl_1	in 8(*j*)	0.1692	0.1650	0.16_7	0.24_0	0.11_9	0.0042 = 0.05
8 Cl_2	in 8(*j*)	0.0375	0.0492	0.04_3	0.24_1	0.35_2	0.0117 = 0.14
4 Cl_3	in 4(*i*)	0.2903	0.2937	0.29_2	0	0.36_8	0.0034 = 0.04
4 $(NH_4^+)_1$	in 4(*i*)	0.2097	0.1875	0.19_9	0	0.63_3	0.0222 = 0.27
4 $(NH_4^+)_2$	in 4(*i*)	0.4113	0.3931	0.40_2	0	0.12_9	0.0182 = 0.22

From the values of $\triangle x$ we obtain an approximate estimation of the accuracy of the x-parameters, and it seems probable that the accuracy of the y- and z-parameters is about the same if we measure them in Å units. The maximum value of $\triangle x_{Cl}$ is 0.14 Å, which seems to indicate that the Sb—Cl distances are accurate to within $\pm$ 0.25 Å, and the Cl—Cl distances to within

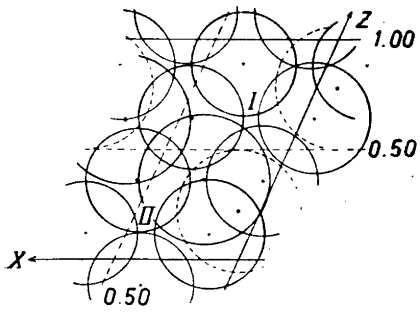

Fig. 9. Cut at $y = 0$ to locate NH_4^+. + projection of centre of Cl. ⊙ projection of centre of Sb. Full circle = forbidden area around Cl and dotted circle = forbidden area around Sb. There are room for the $8NH_4^+$ in two fourfold positions inside the areas marked I and II.

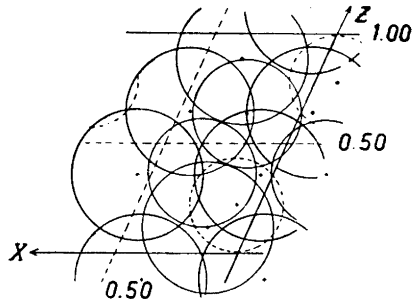

Fig. 10. Cut at $y = 1/4$ to locate NH_4^+. + projection of centre of Cl. ⊙ projection of centre of Sb. Full circle = forbidden area around Cl and dotted circle = forbidden area around Sb. Here is no room for NH_4^+.

$\pm$ 0.5 Å. Considering, however, that the distances Sb—Cl_1 and Sb—Cl_2 are equal (*cf.* Table 3) the real accuracy might be a little better than that stated above.

Because the parameters of the eight NH_4^+ are the least accurate, it is advisable also to ascertain their possible positions from space considerations. They can be situated either in two fourfold positions or in one eightfold position. The sum of ionic radii are NH_4^+—Cl^- 3.24 kX (Goldschmidt, from Internationale Tabellen [46]). Not knowing much about the minimum distance between antimony and ammonium, we assumed it could not be shorter than 3 Å. Considering that the *b*-axis is 7.70 Å, and that there are mirror planes at $y = 0$ and $\frac{1}{2}$, the only possible *y*-parameters are $y = 0$, $y = \frac{1}{2}$, and y approximately 1/4. Spheres of appropriate radii, inside which no ammonium centers could occur, were considered around the chlorine and antimony centers. Cuts were made at $y = 0$, see Fig. 9, and at $y = 1/4$, see Fig. 10. We see that there is only room for the ammonium ions in the cut $y = 0$ inside the areas marked I and II. As the ammonium positions found from the Fourier projections fall inside these two areas, it seems justified to assume these positions. As the exact NH_4^+—Cl distances are not of any particular interest in this case, we did not try to refine the ammonium parameters any further.

F-values were then calculated as

$$F_{calc} = \text{const} \left[f_{Sb} A_{Sb} + f_{Cl} \sum_1^3 A_{Cl_i} + f_N \sum_1^2 A_{NH_4^+} \right]$$

where f_{Sb}, f_{Cl} and f_N are the atomic scattering factors of antimony, chlorine and nitrogen with due consideration taken to their variation with Θ [46]. The calculated and observed *F*-values are given in Tables 1 (for *h0l*) and 2 (for *hk*0).

The reliability factor $R = \dfrac{\sum \big| |F_o| - |F_c| \big|}{\sum |F_o|}$ is 0.24 (0.27 without NH_4^+) for *h0l* and 0.23 (0.27 without NH_4^+) for *hk*0 when only the observed reflections have been included. The values of *R* calculated with an without the NH_4^+-parameters also indicate that these positions are probable.

DISCUSSION OF THE STRUCTURE

As mentioned on p. 129 Sb is surrounded by five Cl at the corners of a deformed octahedron, where the sixth corner is unoccupied, giving an isolated $SbCl_5^{2-}$ group, see Fig. 11. This type of coordination has been postulated by Pauling [47] for an atom with five bonds and one unshared electron pair, which occupies the sixth corner. The distances in the $SbCl_5^{2-}$ group are: Sb—4Cl (approximately in one plane) 2.6_2 Å and Sb—Cl (opposite the empty corner) 2.3_6 Å. We see that the Sb—Cl distance opposite the empty corner is shorter than the others and that the difference is perhaps too large to be explained entirely by the errors in the distances. As a comparison we have the following Sb—Cl distances in the solid state: in Rb_2SbCl_6 2.47 $\pm$ 0.03 kX [26], in SbOCl 2.2_9 and $2.4_7 \pm 0.3$ Å [2]. As to the distances in the gasphase see p. 122.

Table 1. Calculated and observed F-values of h0l for a

h	h00		h01		h02		h03		h04		h05		h06		h07	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
12	31	17	48	22	—36	35	—57	41								
10	13	28	52	53	16		—35	35	12		51	49				
8	—65	61	3		51	54	—85	69	—25	32	68	44	35	17	41	17
6	—35	46	3		71	79	9	17	—86	76	—45	44	22	32	43	33
4	—38	39	—128	150	103	126	109	130	—10	17	—10	17	—74	68	—10	
2	22	30	—122	129	—98	108	42	60	72	76	—4		—18	36	—16	17
0					—97	93	—12		—62	80	36	50	—2	14	—90	95
—2			92	55	6		—27	30	12	14	50	54	38	47	—76	78
4			34	32	—58	62	14	22	—56	61	—2		164	148	9	
6			17	14	58	76	24	37	—61	74	—100	106	9		68	80
8			4		—4	25	86	124	73	103	—105	136	—52	140	29	22
10			—47	55	—71	77	—10		70	88	27	25	—38	58	13	
12			59	65	—25	48	—94	86	39	54	19		—44	30	8	
14			42	41	31		26	33	4		49	44	5		—28	30

Table 2. Calculated and observed F-values of hk0 for a Weissenberg photograph of $(NH_4)_2SbCl_5$. CuKa-radiation.

h	h00		h10		h20		h30		h40		h50		h60		h70		h80		h90	
	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
0					26	28			158	183			21	30			108	64		
1			64	64			41	39			56	58			20	14			49	27
2	30	25			— 3	10			24	26			1				18			
3			6	10			8				1	17			9				— 7	
4	—28	39			—25	32			—23	26			—19	25			—17			
5			—63	69			—63	57			—47	47			—54	42			—33	22
6	—33	57			—90	81			—28	32			—70	55			—24	17		
7			—32	46			—31	32			—25				—14	10				
8	—62	72			— 7				—54	49			1				—43	27		
9			— 6				5				—16	14			13					
10	12	35			84	86			12	17			72	52						
11			48	54			44	58			38	30								
12	25	17			28	32			27	14										
13			30	27			31	35												

Weissenberg photograph of $(NH_4)_2SbCl_5$. CuKa-radiation.

*h*08		*h*09		*h*010		*h*011		*h*012		*h*013		*h*014		*h*015	
F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o	F_c	F_o
—39	36														
30		—34	32												
56	58	—56	52	—25	17	59	20								
52	57	22	20	—82	60	—38	17	26	14	32	17				
71	68	90	82	—14	14	—22	14	—63	40	—14	17				
—90	91	40	44	55	54	11		—1		—26	20	27	28		
—82	63	—21	20	—31	25	30	37	16	14	—58	47	40	28	68	26
7		—21	20	9		12		27	26	—47	36	—79	51	35	26
—18		15		—24	51	—8		97	78	13		—70	37		
37	47	—1		—33	20	—75	66	—2		84	63				
—1	17	30	71	64	48	—75	67	—52	20						
—48	40	—15		62	47										

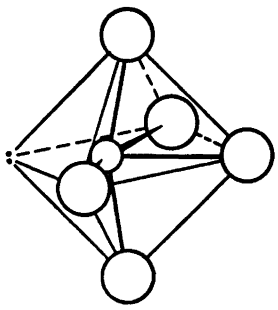

Fig. 11. The $SbCl_5^{2-}$ group with the assumed stereochemically active unshared electron pair occupying the sixth corner of the octahedron.

The $SbCl_5^{2-}$ group is isotypic with the SbF_5^{2-} group in K_2SbF_5[22], where, however, the Sb—F distance opposite the empty corner seems to be larger than the others. For the similarly five coordinated antimony in the complex $Sb_4F_{16}^{4-}$ in $KSbF_4$[23] this Sb—F distance is the shortest. If we interpret the coordination around one of the antimony atoms in Sb_2S_3[16,17] as a five coordination of this sort we find the Sb—S distance opposite the empty corner to be the shortest. An analogous coordination is also found in TlI [48] around both Tl and I, although we have not isolated complexes here. In this case the distance opposite the empty corner is the shortest.

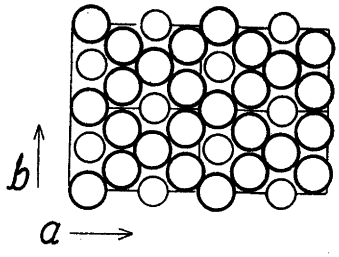

Fig. 12. Approximately close-packed layer at $z \approx 0.36$ projected orthogonally on the ab-plane with four adjacent unit cells. The radii of the circles are equal to the ionic radii of Cl^- (1.81) and NH_4^+ (1.43).

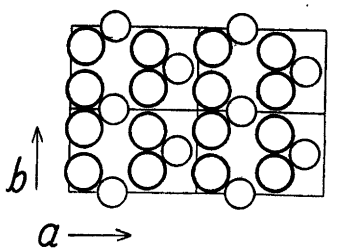

Fig. 13. Approximately close-packed layer with vacant positions at $z \approx 0.12$ projected orthogonally on the ab-plane with four adjacent unit cells. The radii are equal to the ionic radii of Cl^- (1.81) and NH_4^+ (1.43).

We can describe the structure of $(NH_4)_2SbCl_5$ schematically in the following way. The Cl^- and NH_4^+ form together a distorted close-packing with some vacant positions. In this packing we have two types of layers: A, with all positions occupied, see Fig. 12; B, with vacant positions, see Fig. 13. The sequence of the layers is: A,A′,B,B′,A,A′,B,B′, ... These layers are approximately parallel with the *ab*-plane. The Sb atoms are situated in some of the octahedral holes in such a way that one Sb is surrounded by five Cl and with the unshared electron pair directed at the empty position in the close-packing. Orthogonal projections of the structure of $(NH_4)_2SbCl_5$ are given in Fig. 14. The interatomic distances are given in Table 3.

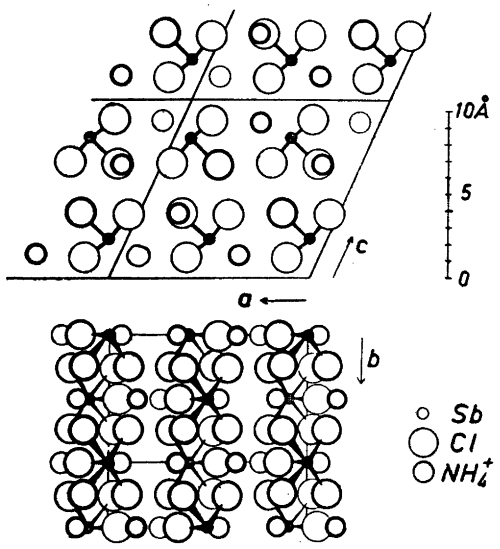

Fig. 14. Orthogonal projections of the structure of $(NH_4)_2SbCl_5$. Parts of adjacent unit cells are given.

Table 3. Interatomic distances in $(NH_4)_2SbCl_5$. Å units. The numeral 2 × before a distance means that it occurs twice.

	In $SbCl_5^{2-}$ group.							
Sb — 5 Cl	$2 \times 2.6_2$			$2 \times 2.6_2$	2.3_6			
Cl — Cl	3.7_0	3.7_2		$2 \times 3.6_6$	$2 \times 3.2_9$	$2 \times 3.4_6$		
Sb — 4Cl (at the "empty" side of the group)	$2 \times 4.3_4$	$2 \times 5.0_6$						
$(NH_4^+)_1$—11 Cl	$2 \times 3.3_9$	$2 \times 3.5_1$	$2 \times 3.6_1$	$2 \times 3.6_2$	3.7_7	$2 \times 3.8_6$		
$(NH_4^+)_2$—9 Cl	$2 \times 3.1_9$	$2 \times 3.3_2$	$2 \times 3.3_8$	3.6_1	$2 \times 3.8_0$			
Cl_1—Sb	2.6_2							
Cl_1—4 NH_4^+	3.1_9	3.3_2	3.3_8	3.3_9				
Cl_2—Sb	2.6_2							
Cl_2—4 NH_4^+	3.1_9	3.5_1	3.6_1	3.6_2				
Cl_3—Sb	2.3_6							
Cl_3—4 NH_4^+	3.6_1	3.7_7	$2 \times 3.8_6$					
Cl_1—7 Cl	3.2_9	3.6_6	3.7_0	3.8_3	4.0_0	4.0_9	4.1_9	
Cl_2—8 Cl	3.4_6	3.6_6	3.6_8	3.6_8	3.7_2	3.9_8	4.0_9	4.1_9
Cl_3—8 Cl	$2 \times 3.2_9$	$2 \times 3.4_6$	$2 \times 3.6_8$	$2 \times 3.6_8$				
$(NH_4^+)_1$—$2NH_4^+$	4.4_4	4.4_8						
$(NH_4^+)_2$—$2NH_4^+$	4.4_4	4.6_0						
Sb—Sb	4.8_1							
Sb—7 NH_4^+	$2 \times 4.3_4$	4.3_5	4.4_3	4.5_4	$2 \times 4.5_6$			
$(NH_4^+)_1$—4 Sb	4.4_3	4.5_4	$2 \times 4.5_6$					
$(NH_4^+)_2$—3 Sb	$2 \times 4.3_4$	4.3_5						

The NH_4^+ ions have a fairly irregular coordination with most of the NH_4^+-Cl distances longer than the sum of the ionic radii, which is 3.24 kX[46]. The long distances might perhaps be explained by the fact that NH_4^+ and Cl^- form a sort of close-packing although they have not the same size.

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The Crystal Structure of Ni_3P. (Fe_3P-Type)

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The structure of Ni_3P (Fe_3P-type) has been determined using single crystal data. The space-groups is $S_4^4—I\bar{4}$ and all atoms occupy general positions 8(*g*). The structure is rather close-packed, resulting in high coordination numbers. The phosphorus atoms have 9 nickel neighbours at an average distance of 2.30 Å. The relation to some other types of transition metal phosphides is discussed and the similarity between the Mo_5Si_3-phase (usually described as "Mo_3Si_2") and the Fe_3P-type is noted.

The first X-ray crystallographic investigation of Fe_3P was made by Hägg [1]. The unit cell was found to be bodycentered tetragonal with the axial lengths $a = 9.090$ kX and $c = 4.446$ kX. The cell contained 8 formula units and the Laue symmetry was $C_{4h}—4/m$. The appearence of 0,0,2 excluded all space-groups except $S_4^2—I\bar{4}$, $C_4^5—I4$ and $C_{4h}^5—I4/m$. The first of these groups was considered to be the most probable one, owing to the external form of the crystals.

In more recent investigations the following isomorphous phases have been found: Mn_3P[2], Cr_3P[3], Ni_3P[4], Mo_3P[5], V_3P[6] * and Ti_3P[7]. A determination of the Fe_3P-structure was, therefore, considered to be of some interest. Ni_3P was chosen for this investigation, because it permitted the use of Cu*K*-radiation without disturbance from fluorescent radiation.

EXPERIMENTAL

The preparation of Ni_3P was made according to the method, described by Haughton [8] and Hägg [1]: Pastilles of red phosphorus were dropped into molten nickel. The nickel was molten in a N_2-atmosphere in a high frequency induction furnace. After cooling the melt was crushed in a steel mortar. Powder photographs were taken in a camera of the Guinier type using Cu*Ka* radiation. Some crystal fragments were selected and the one that gave the best reflexions was used in the single crystal work. For the Weissenberg photographs Mo*K* radiation was used. The crystal was rotated around the *c*-axis and the layer lines 0—4 were recorded. With the aid of Lu's [9] curves, relative $|F|^2$-values were calculated

* Schönberg [12] has not been able to verify the existence of this phase. It seems probable, however, that it does exist.

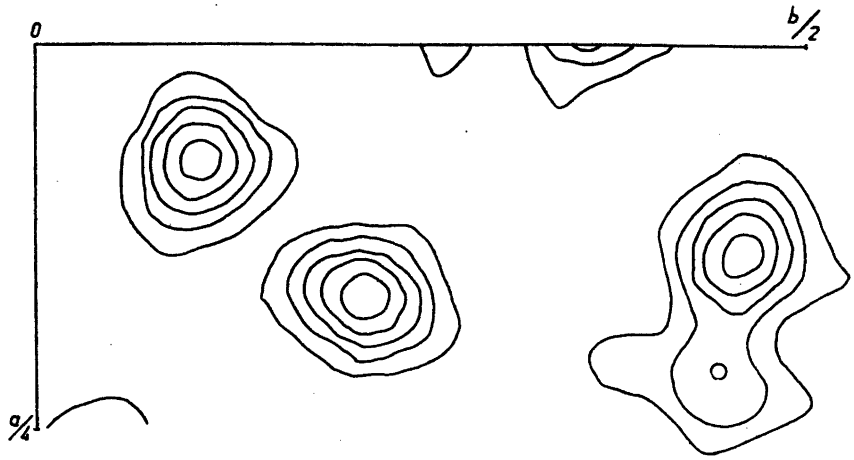

Fig. 1. Final Fourier projection $\varrho x,y$. Contours drawn at intervals of approximately 15e Å^{-2}. 1/8 of the unit cell is given.

from the visually estimated intensities. No corrections were made for adsorption and thermal movement. The computations of the Patterson sections $P(x,y,z_n)$ (z_n = 0, 2/60, 5/60, 10/60, 15/60) and the Fourier projection $\varrho(x,y)$ were made on the Hägg-Laurent machine [10].

DETERMINATION OF THE STRUCTURE

The dimensions of the bodycentered tetragonal cell were determined from the powder photographs. The lengths of the axes are $a = 8.952$ Å and $c = 4.388$ Å ($\pm$ 1 ‰). The Weissenberg photographs showed that $F_{khl} \neq F_{khl}$ and as $F_{0,0,2} \neq 0$ the only possible spacegroups were S_4^2—$I\bar{4}$, C_4^5—$I4$ and C_{4h}^5—$I\frac{4}{m}$. An inspection of the Patterson section $P(x,y,0)$ immediately showed that the fourfold axis could not be a rotation axis. Thus, the spacegroup was found to be S_4^2—$I\bar{4}$— in agreement with the supposition made by Hägg [1]. A further analysis of the Patterson function gave the approximate Ni-positions, which corresponded to general positions 8(g). Successive Fourier-projections $\varrho(x,y)$ refined the x and y parameters of the Ni-atoms and in addition gave those of the P atom, which was also found to occupy a general position 8(g). The conspicuous similarity between the layer lines 0 and 4 as well as the layer lines 1 and 3 indicated that all the atoms must lie very near the planes $z = 0, \frac{1}{4}, \frac{1}{2}$ and $\frac{3}{4}$. This fact was confirmed by the Patterson function, where all the maxima were found in the sections $P(x,y,0)$ and $P(x,y,\frac{1}{4})$.

Fig. 1 shows the final Fourier projection from which the final coordinates were obtained. The maximum corresponding to the P atom is very flat and does not permit an accurate determination of the parameters of this atom. In order to obtain these, different positions of the P atom were tried, until the best agreement between observed and calculated intensities was obtained.

The resulting parameters are

		x	y	z
Ni_I	in 8(g)	0.080	0.109	0.25
Ni_{II}	in 8(g)	0.363	0.030	0
Ni_{III}	n 8(g)	0.164	0.220	0.75
P	n 8(g)	0.290	0.042	0.50

Assuming these parameters to be correct all the maxima of the Patterson function can be explained.

THE STRUCTURE

The structure may be considered as rather close-packed, resulting in high coordination numbers. The environment of each atom is given in Table 1.

One of the Ni-atoms has 12 nickel neighbours (as in pure nickel) but the other two have 10 neighbours. The intermetallic distances are on the average 8 % longer than in the pure metal. The weakening of the metal-metal bonds in comparison with pure nickel is compensated by the metal-phosphorus bonds created here.

The phosphorus atom has 9 nickel neighbours at an average distance of 2.30 Å. Half on the P atoms in Fe_2P[11] and the isomorphous Ni_2P[4] have a similar environment — even the spatial arrangement of the 9 metal neighbours around the P atom is almost the same.

By the determination of the Fe_3P-structures another type of transition metal phosphide has been added to those recently discussed by Schönberg [12]. The way in which an increase of the non-metal concentration results in a decrease of the metallic properties is illustrated by the sequence Fe_3P, Fe_2P and FeP. The coordination numbers become lower. The P atom, for example, has 9 close metal neighbours in Fe_3P. In Fe_2P half of the P-atoms have also 9 neighbours, while the other half have 6, as have the P atoms in FeP. The structures become less densely packed. Assuming $r_{Fe} = 1.26$ Å and $r_P = 1.06$ Å, the volume occupied by each atom in Fe_3P is 6.35 r^3, in Fe_2P 6.8 r^3 and in FeP 7.25 r^3. (The corresponding values are 6.16 r^3 for α-Fe and 5.66 r^3 for γ-Fe.) The shortest P—P distances, being 3.9 Å, 3.6 Å and 2.6 Å, become smaller.

As was mentioned in the introduction several phosphides of the Fe_3P-type have been found among the transition metals (represented in the phosphide systems of Ti, V, Cr, Mn, Fe, Ni and Mo). As the experimental difficulties make

Table 1. (Only distances < 3Å have been given.)

	Interatomic distances in Å
Ni_I — 12 Ni	2.44; 2.52 (2); 2.56 (2); 2.72; 2.79 (5) 2.86
— 2 P	2.25 (2)
Ni_{II} — 10 Ni	2.43; 2.50; 2.54; 2.71 (2); 2.83 (4); 2.86
— 4 P	2.29 (3) 2.32
Ni_{III} — 10 Ni	2.43; 2.52 (3); 2.71; 2.75 (4) 2.78
3 P	2.25 (2) 2.44
P — 9 Ni	2.25 (4); 2.29 (3); 2.33 2.44

the preparation of these compounds rather difficult, it is too early to say definitely which factors determine the occurrence of the Fe_3P-type phosphide. Evidently, it is only formed by the transition metals, belonging to the groups IV—VIII of the periodic system, and most readily by the lightest of those — the only exception between Ti and Ni being Co. In Co_2P, the structure of which has been determined by Nowotny [13], the P atoms have a very similar environment to that of the P atoms in Fe_3P — according to Nowotny the average of the 9 Co—P distances is 2.30 Å. There is no Cu-compound of this type. Cu_3P has a different structure which has been determined by Steenberg [14]. To what extent the Fe_3P-type phosphide is formed by the heavier transition elements cannot yet be stated with certainty.

Another question is whether any other non-metal forms a similar phase with the transition metals. The Mo—Si and W—Si systems have recently been investigated at this institute and the phases usually described as "Mo_3Si_2" and "W_3Si_2" have been found to possess a bodycentered tetragonal structure with $a = 9.617$ Å, $c = 4.899$ Å ("Mo_3Si_2") and $a = 9.645$ Å, $c = 4.969$ Å ("W_3Si_2"). The correct formulae are Mo_5Si_3 and W_5Si_3 with 4 formula units in the elementary cell. Thus, the elementary cell of this structure resembles very strikingly that of Fe_3P. A structure determination of W_5Si_3, which has recently been completed at this institute, shows, however, that this similarity is not caused by any deeper analogies between the Fe_3P and W_5Si_3 structures. (The space group of W_5Si_3 is $I\bar{4}2m$; 16 W are situated in 16 (j) with $x = 0.277$, $y = 0.074$, $z = 0.25$, 2 W in 2 (b), and 2 W in 2 (a), while the probable positions of Si are 8 Si in 8 (i) with $x = 0.17$, $z = 0.75$ and 4 Si in 4 (c)).

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The Crystal Structures of the Isomeric Squalene Hexahydrochlorides

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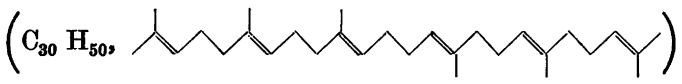
The structures of two isomeric squalene hexahydrochlorides have been investigated by the aid of X-ray methods, supported by the study of the infrared absorption spectra. Detailed molecular structures are proposed for both forms, and their relation to other long carbon chain molecules such as β-guttapercha, rubber hydrochloride and geranylamine hydrochloride is discussed. It is shown that the reason for the isomerism lies in the steric orientation of chlorine atoms and methyl groups at the four asymmetric carbon atoms, and the two crystalline isomers have been identified as the two possible *meso*-forms of the molecule. These structure determinations constitute an independent proof of the accepted structure of the carbon skeleton of the squalene molecule.

Both natural and synthetic squalene



upon saturation with hydrogen chloride yield a mixture of crystalline hexahydrochlorides, which can be separated into two fractions, one of which constitutes about 80 % and melts at 108—110° C, while the other one constitutes about 20 % and melts at 143—145° C (see for instance Karrer and Helfenstein [1]). Sometimes two low melting isomers have been reported in the literature. We have found, by repeated crystallizations, that all crystal crops with deviating melting points are mixtures of the higher melting form and the lower melting form mentioned above (in this paper designated by h.m. form and l.m. form, respectively).

Both isomers have the same composition ($C_{30}H_{56}Cl_6$) and give very similar, though not identical, X-ray diffraction patterns. Nevertheless, they are distinctly different as evidenced by their melting points, solubility properties, specific gravities, X-ray data and infrared absorption spectra. No attempt to clarify the reason for this isomerism has hitherto been made. It could scarcely be connected with isomerism of the parent hydrocarbon for several reasons:

1) Regeneration of the hydrocarbon from both isomers gives indistinguishable products; 2) Both these products, and also synthetic squalene made by different methods, give with hydrogen chloride the same mixture of hexahydrochlorides as obtained from squalene itself, although these hydrocarbons differ from natural squalene with respect to double bond orientation around at least some of the substituted carbon atoms (*cf.* Dauben *et al.*[2]).

It would appear that the only reasonable cause for this isomerism is to be found in the steric orientation of the chlorine atoms and methyl groups at the four asymmetric carbon atoms of the chain, making the fair assumption that chlorine adds to the methylsubstituted carbons according to Markownikoff's rule. The only difficulty is that out of the 6 possible diastereoisomers only two are obtained in crystalline form and in so widely different quantities. Evidently, the addition mechanism must favour the formation of the lower melting isomer. Thus, the structure determination of these crystal modifications is of interest from two points of view, *i. e.*, the stereochemistry of polymeric carbon chains, and the mechanism of the addition of hydrogen chloride to unsaturated hydrocarbons.

The present paper reports the results of X-ray investigations on the structures of the two isomers of squalene hexahydrochloride, supported by chemical and optical observations.

UNIT CELLS AND SPACE GROUPS

Both isomers of squalene hexahydrochloride crystallize in thin transparent plates with perfect cleavage parallel to the plane of the plates. Cleavage in several directions perpendicular to these planes is also observed, giving the small plates a pseudo-hexagonal cross-section. This is most pronounced for the higher melting form, which also looks more homogeneous under the microscope in polarized light than the lower melting one. Both isomers show sharp extinction in polarized light, from which the lack of optical activity may be inferred. No difference can be detected in their X-ray powder photographs. The unit cell dimensions and rules for systematic absences were determined from oscillation and Weissenberg photographs obtained with Cu$K\alpha$-radiation.

For the h.m. form: $a = 56.7_0 \pm 0.1$ Å; $b = 10.23 \pm 0.02$ Å; $c = 5.99_2 \pm 0.01$ Å; $\gamma = 93.2° \pm 0.2°$.

$V = 3\,468$ Å^3, $d_{calc.} = 1.205$ g/cm^3, $Z = 4$ mol./u.c.

For the l.m. form: $a = 56.7_1 \pm 0.1$ Å; $b = 10.40 \pm 0.02$ Å; $c = 5.98_4 \pm 0.01$ Å; $\gamma = 92.0° \pm 0.2°$.

$V = 3\,526$ Å^3, $d_{calc.} = 1.185$ g/cm^3, $Z = 4$ mol./u.c.

Thus, both isomers are monoclinic and pseudo-orthorhombic, but only very small differences are revealed in their cell dimensions. The small but significant difference in specific density has been confirmed by determinations by the floating method, which gave 1.20_8 and 1.18_2 for h.m. and l.m. form, respectively.

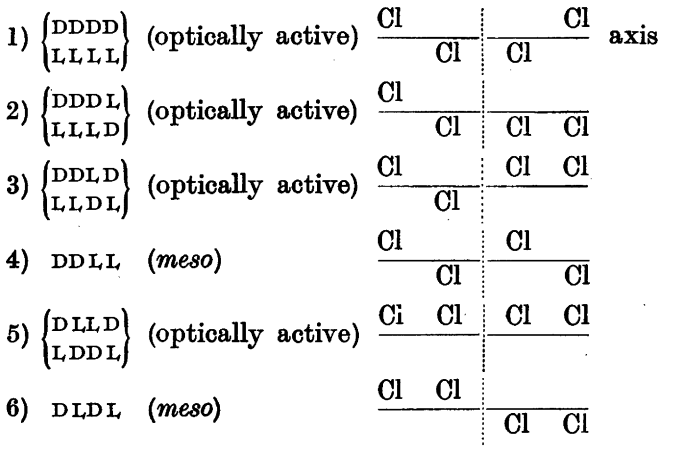
The same systematic absences and pseudo-absences are found for both forms:

$h00$ for h odd; $0k0$ for k odd; $00l$ for l odd; $h0l$ for $h + l$ odd; $hk0$ for k odd.

Since the angle γ deviates from 90° for both forms, only the third and the fifth rule could be strictly valid, the other rules must be due to pseudo-symmetries, but it may be of interest to note that all these rules together would indicate the orthorhombic space group *Pnma* (D_{2h}^{16}) (with the *a*-axis and the *b*-axis interchanged). The systematic absences $00l$ for l odd and $hk0$ for k odd indicate a screw axis parallel to [001] and a glide plane parallel to (001) with translation $b/2$. This corresponds to the space group $P2_1/b(C_{2h}^5)$.

POSSIBLE ISOMERIC STRUCTURES

Six possible diastereo-isomers of squalene hexahydrochloride, four D,L-forms and two *meso*-forms, may be anticipated from the disposition of the chlorine atoms and methyl groups. These six possible isomers may be visualized schematically in the following way:



To the left are given the sequences of the four *asymmetric* orientations, to the right the resulting orientations in the crystal lattice. The horisontal line represents the approximate "plane" of the folded carbon zig-zag chain, which will fit with the length of the unit cell axis (*a*) and which has been shown by Bunn and Garner [3] to exist in the structure of rubber hydrochloride in crystalline form. The chlorine atoms will then have to lie either above or below this "plane".

THE DETERMINATION OF THE STRUCTURES

The Weissenberg photographs of the equatorial layer lines around the principal axes of the crystals show fairly sharp and well-defined reflexions, especially the $h00$-reflexions, for which even orders until the 62nd are observed. Most of the $hk0$-reflexions are very weak, and only very small differences are observed between the intensities for the h.m. form and the l.m.form. The glide plane of symmetry implies that the x-coordinates for corresponding atoms of molecules, lying beside each other in the *b*-axis direction, are the same. This means that a fairly good resolution of the electron density variation might be expected in a Fourier projection on the crystallographic *a*-axis direction. Clear differences in intensities might probably be expected first for the hkl-reflexions. These, however,

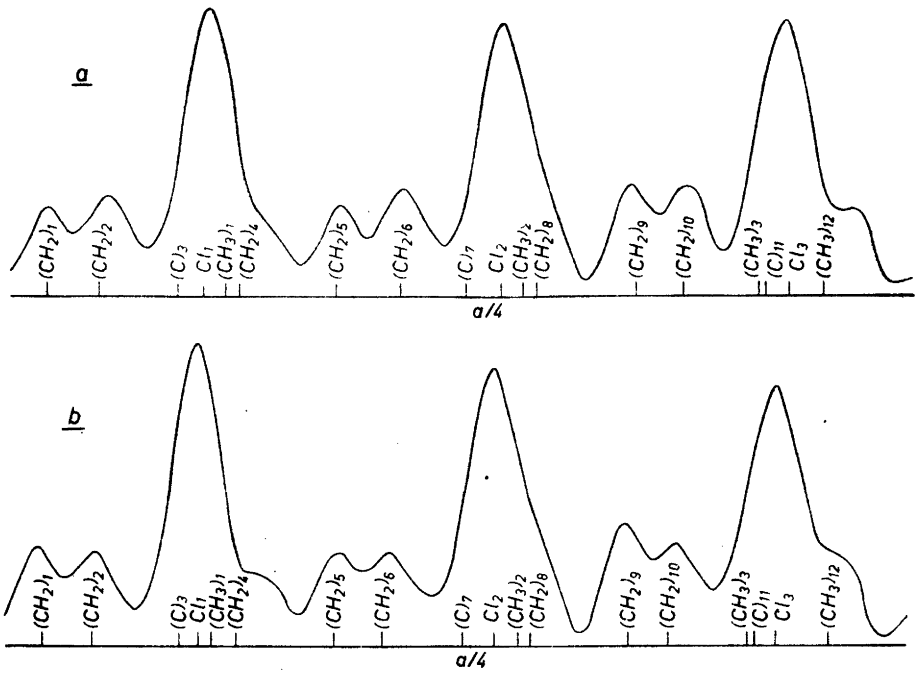


Fig. 1. Projections of electron density on [100] *a) for the higher melting form, b) for the lower melting form of squalene hexahydrochloride. Atomic co-ordinates, as derived by the method of least squares, are indicated.*

would in an ordinary Weissenberg photograph with Cu$K\alpha$ radiation be separated only by about 0.5 mm, and it was in fact found that the reflexions on higher levels are smeared out into lines, making the determinaticn of intensities difficult and uncertain.

In this position it was found that the best way of attacking these structures would be to obtain a maximum of informations from one-dimensional projections of electron densities on [100]. The intensities of the $h00$-reflexions were estimated visually, using a graduated intensity scale of reflexions with approximately the same size and form as those to be determined. The reading of the intensities were repeated five times on different photographs, and corrected in the usual way, the absorption error being neglected since the crystals used were very small (0.1—0.2 mm).

The successive determinations of the signs for the $h00$-reflexions, by means of a direct method to be described elsewhere, led to the projections of electron densities on the long axis for the h.m. form and the l.m. form as shown in Fig. 1. Differences in structure for the two forms could hardly be deduced from these projections, because of the strong overlapping of the densities for the chlorine atoms and the nearest carbon atoms. The carbon skeleton of the chain and the positions of the chlorine atoms and methyl groups were, however, at once evident from these projections. In order to bring out some possible differences between the structures of the two forms, and possibly also a decision for one or the other of the 6 isomeric structures discussed above, both series of x-coordinates were subjected to repeated least squares refinements. This treatment brought out some conspicuous differences in the projected bond lengths, especially for the C—C-bonds on the central sides of the asymmetric carbon atoms. The final x-coordinates and the projections of the bond lengths along the carbon chain are set out in Table 1. The observed and calculated structure factors are listed for both forms in Table 2. The reliability index is 0.126 for the h.m. form and 0.115 for the l.m. form.

THE STRUCTURE OF THE SQUALENE HEXAHYDROCHLORIDES

In order to reconstruct the skeleton of the carbon chain from the knowledge of the projected C—C distances it was necessary to make the assumption of 1.54 Å for the C—C bond length. This value may, however, be considered as very well established and there should be no reason to expect any remarkable deviation from this value in the present case. Secondly, the assumption of 114° for the angle between the bond directions at the chain carbons was preliminarily introduced. In the course of the calculation this value can be corrected, and it was found that the present data would suggest a value of about 115° for the bond angles, with the exception of those at the tetrasubstituted carbons, for which values of 108—109° would fit better with the present observations. The angle ε between a C—C bond and the a-axis direction is calculated for both forms and may be found in Table 1.

There might be some doubt as to the sequence of chain carbons and methyl groups in the projection, for example, whether the fourth or the fifth carbon should be ascribed to the methyl group. If, however, the fifth one were assumed to be the methyl group, the fourth one being a chain carbon, the projection of the C—C bond length of the chain would be 1.73 Å for the h.m. form and 1.93 for the l.m. form, whereas the same projected bond lengths come out to be 1.52 Å and 1.54 Å, respectively, if the fourth carbon belongs to the methyl group and the fifth to the chain. It seems justified to consider these data as a decisive proof of the sequence —C*—Cl—CH_3—CH_2— in the projection, and a similar argument indicates the same sequence at the second

Table 1. Atomic co-ordinates (Θ_1) in degrees, projected bond-lengths on the a*-axis in Å, and angle between bond and* a*-axis for the squalene hexahydrochloride isomers. The middle point of a molecule has been chosen as origin.*

Atom or group	Θ_1 in degrees		Chain carbon distance	Projected bond-length (P_x) in Å		Angle (ε) between bond and a-axis	
	h.m. form	l.m. form		h.m. form	l.m. form	h.m. form	l.m. form
Cl_1	38.7	38.6	$C\bar{l}-C_1$	1.19	1.16	39°	41°
Cl_2	97.7	97.8	C_1-C_2	0.82	0.79	58	59
Cl_3	154.3	154.5	C_2-C_3	1.22	1.38	*37*	*26*
$(CH_2)_1$	7.6	7.4	C_3-C_4	0.97	0.83	51	57
$(CH_2)_2$	18.0	17.5	C_4-C_5	1.53	1.53	·0	0
$(C)_3$	33.5	35.1	C_5-C_6	0.98	0.77	50	60
$(CH_2)_4$	45.8	46.6	C_6-C_7	1.05	1.24	*47*	*36*
$(CH_2)_5$	65.2	66.0	C_7-C_8	1.10	1.05	44	47
$(CH_2)_6$	77.7	75.8	C_8-C_9	1.52	1.54	~0	0
$(C)_7$	91.1	91.6	C_9-C_{10}	0.72	0.65	62	65
$(CH_2)_8$	105.0	105.0	$C_{10}-C_{11}$	1.31	1.34	31	29
$(CH_2)_9$	124.3	124.5	$C_{11}-C_{12}$	0.90	1.12	54	43
$(CH_2)_{10}$	133.4	132.8					
$(C)_{11}$	150.1	149.8					
$(CH_3)_{12}$	161.6	164.3					
$(CH_3)_1$	43.3	41.4					
$(CH_3)_2$	102.2	102.7					
$(CH_3)_3$	148.9	148.1					

tetrasubstituted carbon. Stereochemical considerations will also show that the chlorine atoms and the methyl groups must be connected to the third, eighth and fourteenth carbons of the projection, because the deviations from a planar zig-zag carbon chain are found at these carbon atoms (see below). Furthermore, it is seen that the chlorine atom connected to a tetrasubstituted carbon for both forms, lies closer to the carbon in the projection, than does the methyl group connected to the same carbon.

The structures which emerge from the data given in Table 1 may be described as follows: The four carbons, constituting the central part of the molecule, lie approximately in a plane, which makes an angle of about 40° with the chain direction (*a*-axis), this angle being larger for the l.m. form than for the h.m. form. From the two asymmetric carbons, towards the ends of the molecule, there extend planar groups of four carbons, the planes of these groups being parallel to the *a*-axis. The bonds from the asymmetric carbons, towards the central side, linking the planar groups together, are tilted an angle (ε) of about 30—40° away from the planes of these groups, these angles being appreciably larger for the h.m. form than for the l.m. form. (Strictly ε denotes the angle between a C—C bond and the *a*-axis, but since the planes of the planar groups in question are approximately parallel to the *a*-axis, ε will also approximately indicate the tilting of these bonds with respect to the planar groups). This difference in the tilting of the bonds, linking the planar 4 C-groups together, is the only feature which clearly discriminates between the structures of the two isomeric forms. The result is a more pronounced folding of the carbon chain for the h.m. form than for the l.m. form.

The folding of the chain at each tetrasubstituted carbon atom may be necessary to provide place for the methyl group and chlorine atom, and from molecular models it may also seem likely that the non-planar structure here is mainly due to repulsion between CH_2-groups of the chain.

Only two models can be derived such that they satisfy the requirements of the present observations. First, the bond on the central side of both asymmetric carbons are tilted in the same direction away from the planes of the planar 4 C-groups, the chlorines Cl_1 and Cl_2 must be on the same side of the chain in order to appear on the central side of the methyl group in the projection. Secondly, if these bonds are tilted in opposite direction, the chlorines must, for the same reason, lie on opposite sides of the chain (the argument is, in both cases, based on the assumption of an approximately tetrahedral arrangement of bonds from the asymmetric carbons).

The two structures, which result from these considerations, are indicated in Fig. 2, and may be seen to correspond to models Nos. 4 and 6, *i. e.*, the two *meso*-forms of the six possible diastereoisomers. The other models would correspond to asymmetric molecules, which would give rise to odd orders of the $h00$-reflexions, provided they would be strong enough to be observed. The structure factors F_{h00} are, however, very sensitive to an all over displacement of the atoms. The absence of odd orders of $h00$, therefore, speaks clearly in favour of the two symmetric forms Nos. 4 and 6 above, and consideration of the packing of the molecules points in the same direction. The projection of electron density on [100] can, of course, give no information about the orientation of the molecules around this axis. The packing of the molecules, as well

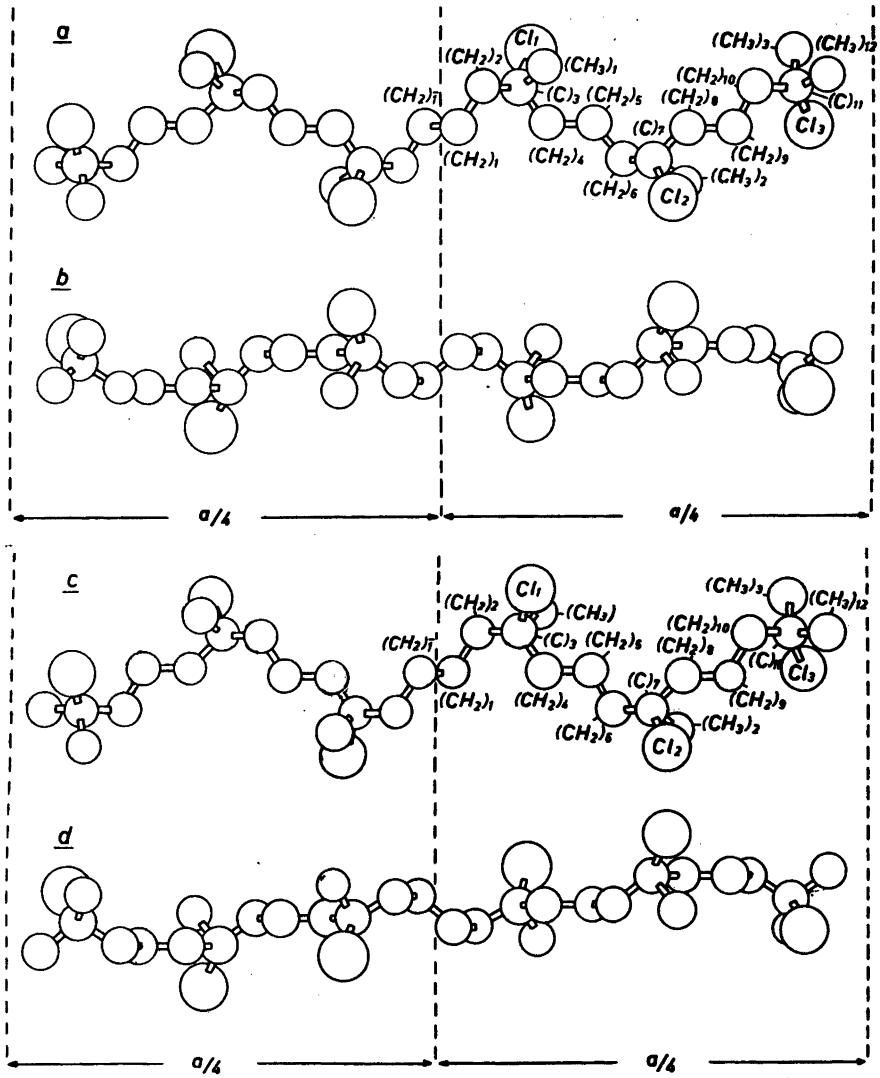


Fig. 2. Molecular structure models for the two crystalline isomers of squalene hexahydrochloride, a) and b) for the higher melting form, c) and d) for the lower melting form. In a) and c) the molecules are viewed along the c*-axis direction, in b) and d) along the* b*-axis direction.*

as comparison with the apparently closely related structure of rubber hydrochloride [3] would suggest that the main plane of the molecule is approximately parallel to the (001)-plane, which implies that the C—Cl-bonds and the C—CH_3-bonds are mainly pointing in the direction of the *c*-axis of the crystal.

As to the orientation of the arrangement around the end tetrasubstituted carbons, this could hardly be deduced with certainty from the obtained pro-

Table 2. Observed and calculated structure factors for the higher melting form and the lower melting form of squalene hexahydrochloride.

*h*00 *	H.m. form		L.m. form	
	$F_{obs.}$	$F_{calc.}$	$F_{obs.}$	$F_{calc.}$
1 0 0	—	−8	—	−9
2 0 0	24	−25	24	−24
3 0 0	—	3	9	4
4 0 0	44	−44	46	−44
5 0 0	45	−53	49	−55
6 0 0	181	−180	163	−166
7 0 0	121	139	125	132
8 0 0	14	13	15	15
9 0 0	54	49	54	47
10 0 0	18	27	25	36
11 0 0	80	71	92	76
12 0 0	32	−14	36	−29
13 0 0	167	−158	168	−164
14 0 0	27	25	16	14
15 0 0	31	−37	33	−32
16 0 0	28	−13	35	−24
17 0 0	22	17	13	13
18 0 0	45	45	44	41
19 0 0	65	56	55	65
20 0 0	40	−46	15	20
21 0 0	14	15	15	17
22 0 0	16	−12	15	−12
23 0 0	12	−12	22	−25
24 0 0	14	−17	28	−30
25 0 0	52	−43	51	−43
26 0 0	15	18	26	23
27 0 0	—	−11	—	−17
28 0 0	—	7	22	29
29 0 0	—	4	—	11
30 0 0	—	9	—	3
31 0 0	35	−42	32	−42

* Strictly, these indices should be doubled.

jections, especially because the arrangement apparently deviates considerably from a regular one. The obtained projections indicate, however, that the end groups could not possibly have a similar orientation as the groups around the other tetrasubstituted carbons, but more likely be turned about 90° away from these orientations; *i. e.*, about 90° around the single C—C bond on the central side of the end tetrasubstituted carbon (see Fig. 2). The direction of the tilting of this bond cannot be derived unequivocally from the one-dimensional projection, but considering the all over directions of these very long chain molecules, the directions indicated in Fig. 2 may reasonably be assumed as the most likely ones.

The question now remains as to which of these structures should be ascribed to the h.m. form and which to the l.m. form. It may then be seen from Fig. 2 that structure model No. 6 has the bonds, connecting the planar 4 C-groups, tilted in the same direction, which implies that the molecule will lie somewhat inclined to the *a*-axis in the crystal. This inclination of the molecules is likely

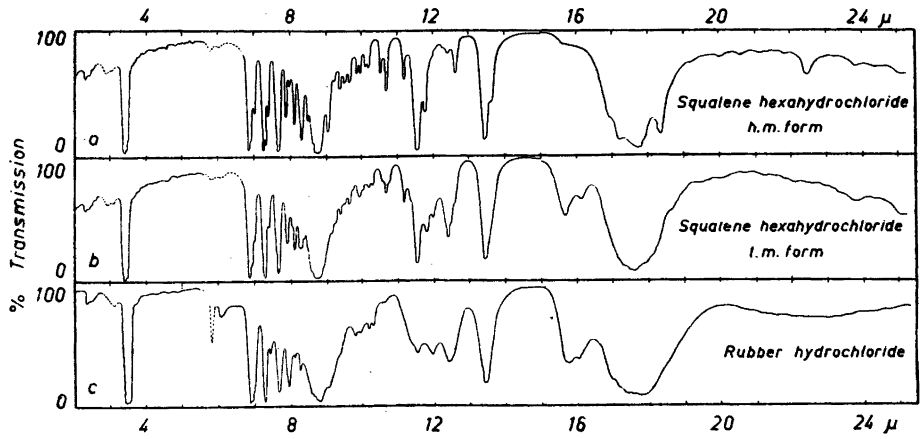


Fig. 3. Infrared absorption spectra from 2—15 μ (NaCl-prism) and from 15—25 μ (KBr-prism) of a) h.m. and b) l.m. squalene hexahydrochloride as pressed KBr-disks and of c) commercial rubber hydrochloride film containing 29 % Cl, or 85 % of the theoretical value.

to reduce the tightness of packing and put a restriction on the deviation from a planar structure. Considering the values of the angle ε in Table 1 for the bonds C_2—C_3 and C_6—C_7, which are 26° and 36° for the l.m. form, against 37° and 47° for the h.m. form, it may be reasonable to suggest that the l.m. form has a structure, corresponding to model No. 6, indicated in Fig. 2 c and d. In structure model No. 4, (Fig. 2) the bonds between planar 4 C-groups are tilted in alternating directions, leaving the main direction of the molecule parallel to the crystallographic *a*-axis. Thus, no strong restriction on the inclination of these bonds is present for this case, and it is likely that the h.m. form has the molecular structure indicated in Fig. 2 a and b. The study of the infrared absorption spectra seems to support this point of view (See next section).

INFRARED ABSORPTION SPECTRA

The infrared absorption spectra of the two squalene hexahydrochlorides are shown in Fig. 3. They were recorded in a Perkin-Elmer double beam instrument, Model 21, as pressed potassium bromide disks. As might be expected the two spectra are rather similar. The most conspicuous differences are the bands at 9.05 μ, 12.6 μ, 18.35 μ and 22.5 μ, present in the h.m. form but absent in the l.m. form, and the bands at 12.4 μ and around 16 μ (15.7 and 16.1 μ) present in the l.m. form but absent in the h.m. form. That these differences are mainly due to differences in crystal structures, is shown by the fact that solutions and undercooled melts give practically identical spectra; thus, the h.m. form most strikingly loses its bands at 18.35 and 22.5 μ and develops a band at around 16 μ. The non-crystallizing mother liquor also gives roughly the same spectrum.

It may be noted that the broad band at 17—18 μ, which is due to the C—Cl stretching vibration and agrees very well with the value 570 cm^{-1} found for *tert*-butylchloride and *tert*-amylchloride [4, 5], and the band (or band group) at 8.5—9 μ, which are the two most prominent bands in both spectra, show more detail in the h.m. form than in the l.m. form. Incidentally, the frequency of the band group at 8.5—9 μ is just twice that of the C—Cl stretching frequency. No overtone is involved, however, since the same strong band is present also in the spectra of the squalene hexahydrobromides.

For comparison the spectrum of a commercial sample of rubber hydrochloride has been recorded (as film) and included in Fig. 3. It is evident that it resembles the l.m. form much more than the h.m. form, although, of course, many details occurring in the squalene hexahydrochloride spectra are lacking in that of rubber hydrochloride. This is taken as a confirmation of the result of the structure determination, indicating that the general shape of the l.m. form, *i. e.* with the chlorines of each half on the same side, is more similar to rubber hydrochloride than is the h.m. form, where the chlorines are alternately on opposite sides and to a greater extent are surrounded by chlorine atoms of neighbouring molecules. The molecules of the h.m. form so to say fit into each other like cog-wheel teeth, and one would expect greater van der Waals forces between molecules, as actually evidenced by tighter packing, higher melting point, lower solubility and thinner plates of the crystals.

DISCUSSION

The present structure determinations of the isomers of squalene hexahydrochloride have been worked out on the basis of a relatively limited amount of experimental data. A two-dimensional projection of electron density on, for example, (001) would possibly give more definite informations, provided that all signs of the numerous weak reflections were obtained with certainty, a task which would involve a vast amount of calculation, and even considerably more if a three-dimensional synthesis were intended. It is reasonable to believe that the intensities of the *h*00 reflexions are fairly accurate, and the method used in deriving the projection of electron density on [100] involved no assumptions as to the structure in beforehand. The present structure determination, therefore, constitutes a direct and completely independent proof of the accepted structure of the carbon skeleton of the squalene molecule. As to the type and location of the double bonds it can only be said from this work that they have to involve the substituted carbon atoms.

The characteristic feature of planar segments of four carbon atoms in the chain connected by C—C bonds at angles of 30—40° is reminiscent of the structures proposed for rubber and β-guttapercha (*cf.* Bunn [6] and Jeffrey [7]) and established in geranylamine hydrochloride by Jeffrey [8]. In these cases the planar isoprene groups are necessitated by the double bond in the middle of each group, whereas the planar groups in the squalene hexahydrochlorides are displaced two carbons along the saturated chain. Molecular models show clearly that the reason for these tilted bonds between planar 4 C-groups is the same in all cases; *viz.* a consequence of steric repulsions between CH_2, CH_3 and CH groups in the former case, and chiefly between CH_2 groups in the latter case. The same structural features are, therefore, to be expected in rubber hydrochloride, in particular since the structure proposed by Bunn and Garner demands unusually large distortions of the tetrahedral arrangement around the substituted carbons (*cf.* the criticism advanced by Jeffrey [7]).

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On Ion Exchange Equilibria

III *. An Investigation of Some Empirical Equations

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Empirical equations by the following authors for representing ion exchange equilibria are discussed: Kroeker, Vageler, Weisz, Boedeker, "Freundlich", Wiegner-Jenny, van Dranen and Rothmund-Kornfeld (*cf.* Table 1). Also an empirical equation recently given by Yamabe and Sato is discussed in a note. Discussion is limited to exchange between monovalent ions except when discussing the Rothmund-Kornfeld equation. It is shown that the linear plots used for determining the constants in the empirical equations are too insensitive to be used for testing the applicability of the equation under consideration. A more sensitive plot is obtained if the constants in the empirical equation are used for calculating the equilibrium quotient curve $\varkappa$ (β). There are three different kinds of equilibrium quotient curves recorded in literature: falling, rising and curves with maximum or minimum (*cf.* Fig. 1). No empirical equation except the one of "Freundlich" is capable of representing all of these types. The most useful equation seems to be that of Rothmund and Kornfeld which can be extended in a very simple manner to all three types of equilibrium quotient curves. In connection with this equation some different methods for finding an approximate value of the thermodynamic equilibrium constant are discussed, and it is shown that $k^{1/p}$ (where k and p are the empirical constants in the Rothmund-Kornfeld equation) and $\varkappa_{B,A(\beta=0.5)}$ may be useful first approximations to the thermodynamic equilibrium constant.

In the earlier work on ion exchange a number of empirical equations were used for representing equilibrium data. Later various forms of the law of mass action were used and, at present, it is very common to represent ion exchange equilibrium data by plotting the equilibrium quotient (see eq. (2) below) against some function of the ionic concentrations. Such plots show that, in most cases, the equilibrium quotient varies. For cases where the variation is large it has been suggested [1] that other expressions *e.g.* some empirical equation, may be more useful. The present paper deals with the possibility of representing variations in the equilibrium quotient by empirical equations.

* For parts I and II see Refs. 7 and 18.

Discussion is limited to cation exchange equilibria and, for the sake of simplicity, to exchange between monovalent ions. Only when discussing the Rothmund-Kornfeld equation will exchange between multivalent ions be considered.

For the ion exchange reaction:

$$B^+ + AR \rightleftharpoons A^+ + BR \tag{1}$$

where A^+ and B^+ are the two exchanging cations and R the anion framework of the exchanger an equilibrium quotient can be defined as follows:

$$\varkappa_{B,A} = \frac{\{A^+\} \cdot [BR]}{\{B^+\} \cdot [AR]} \tag{2}$$

where $\{\}$ denotes activity and [] concentration.

The following mole fractions are used:

$$\alpha = \frac{[B^+]}{[A^+] + [B^+]} = \frac{[B^+]}{a_0}; \quad \beta = \frac{[BR]}{[AR] + [BR]} = \frac{[BR]}{s_0} \tag{3}$$

where a_0 is the total ionic concentration of the solution (which is here equal to the ionic strength) and s_0 is the saturation capacity of the exchanger. From (2) and (3) the following expression for $\varkappa_{B,A}$ is obtained:

$$\varkappa_{B,A} = \frac{f_A(1-\alpha)\beta}{f_B\alpha(1-\beta)} \tag{4a}$$

where f_A and f_B are the activity factors of A^+ and B^+ in the liquid phase. In dilute solutions $f_A = f_B$ and

$$\varkappa_{B,A} = \frac{(1-\alpha)\beta}{\alpha(1-\beta)} \tag{4b}$$

CHOICE OF GRAPHICAL REPRESENTATION

It has proved convenient to plot $\varkappa$ against β, and the three different types of $\varkappa(\beta)$-curves reported in the literature are given in Fig. 1. In the following, curves with $\frac{d\varkappa}{d\beta} < 0$ are referred to as type I, with $\frac{d\varkappa}{d\beta} > 0$ as type II and curves with a maximum or minimum as type III. Hitherto type I has been most usual but examples of the other two types can be found in papers by Argersinger *et al.*[2,3], Bonner and Rhett [4], Duncan and Lister [5,6], Högfeldt *et al.*[7] and Marinsky [8].

The empirical equations used give a simple relation either between the amount of a certain ion added and the amount sorbed by the exchanger at equilibrium, or between the equilibrium concentration in solution and the amount sorbed by the exchanger. In the latter case the empirical equation gives a relation of the type:

$$\alpha = \varphi(\beta) \tag{5}$$

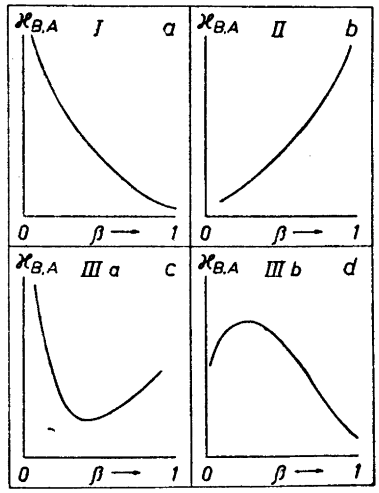

Fig. 1. The three different types of $\varkappa(\beta)$-curves. Fig. 1a represents a falling curve, Fig. 1b a rising curve and Figs. 1c and 1d are examples of curves with a minimum or maximum.

where $\varphi(\beta)$ is a function of β, which inserted in (4b) gives a function $\varkappa(\beta)$ which can be studied and compared with curves obtained experimentally. In the first case a relation between α,β and the total amount of the ion under consideration is required. Such a relation is given by the conservation of mass and takes the following form if the exchanger saturated with A^+ is equilibrated with V litres of solution of B^+ of initial concentration c_0, and A^+ of initial concentration a_0—c_0:

$$\beta s_0 + V\alpha a_0 = Vc_0 \tag{6}$$

From (6) and the empirical equation:

$$Vc_0 = \psi(\beta) \tag{7}$$

where $\psi(\beta)$ is a function of β, an expression for $\varkappa(\beta)$ can be obtained.

In order to be able to use (6) one must make experiments under such conditions that V, a_0 and s_0 are held constant.

The type of curve obtained if $\varkappa_{A,B} = \varkappa_{B,A}^{-1}$ is plotted against $(1-\beta)$ instead of $\varkappa_{B,A}$ against β may now be considered. The three types of $\varkappa(\beta)$-curves are characterized by the sign of $\frac{d\varkappa}{d\beta}$. For $\frac{d\varkappa}{d\beta} < 0$ we have type I, for $\frac{d\varkappa}{d\beta} > 0$ type II and for $\frac{d\varkappa}{d\beta} \gtrless 0$ type III.

The sign of $\frac{d\varkappa}{d\beta}$ is known and the sign of $\frac{d\varkappa^{-1}}{d(1-\beta)}$ is required:

$$\frac{d\varkappa^{-1}}{d(1-\beta)} = \frac{-d(1/\varkappa)}{d\beta} = \frac{1}{\varkappa^2}\frac{d\varkappa}{d\beta}$$

Thus $\frac{d\varkappa}{d\beta}$ has the same sign as $\frac{d\varkappa^{-1}}{d(1-\beta)}$. If $\varkappa(\beta)$ belongs to type I or II, $\varkappa^{-1}(1-\beta)$ belongs to the same type. If, however, $\varkappa(\beta)$ belongs to type IIIa,

$\varkappa^{-1}(1-\beta)$ belongs to type IIIb and *vice versa*. This depends upon the reversal in orientation to the two axes. The corresponding $\varkappa^{-1}(1-\beta)$-curves can be visualised by turning the $\varkappa(\beta)$-curves in Fig. 1 upside down.

It is of course an arbitrary matter which kind of curve one chooses to plot. One conveniently takes B^+ as the most strongly sorbed ion. The equilibrium quotient then becomes larger than unity and the $\varkappa(\beta)$-curve gives the variation of selectivity with concentration for that ion over the other. For curves with a maximum or minimum the distinction between types IIIa and IIIb is only a consequence of this convention as one could equally well plot the reciprocal function and transform IIIa to IIIb, or *vice versa*, and only make use of one of them.

RESULTS

Expressions for $\varkappa(\beta)$ have been calculated as outlined above from the empirical equations given by the following authors: Boedeker [9], van Dranen [10], "Freundlich" [11], Kroeker [12], Rothmund-Kornfeld [13-14], Vageler [15], Weisz [16] and Wiegner-Jenny [17].

In Table 1 the different empirical equations and the corresponding $\varkappa(\beta)$-functions are summarized. From this table it can be seen that the equations of Kroeker and van Dranen contain only one constant whereas all others contain two constants which may be determined from the linear plots indicated in the fourth column. For the equations of Kroeker, Vageler, Boedeker and Wiegner-Jenny one must keep a_0, $\bar{V}$ and s_0 constant in order to be able to use (6) for calculating $\varkappa(\beta)$. The numbers I—III in the last column but one refer to the different types of $\varkappa(\beta)$-curves given in Fig. 1. From the last column but one of Table 1 it can be seen that no empirical equation but the one of "Freundlich" is capable of representing all types of $\varkappa(\beta)$-curves found in the literature. These results suggest the possibility of arranging the empirical equations according to the type of $\varkappa(\beta)$-curve which may be derived from them.

The applicability of the different empirical equations for representing experimental data has been tested by using the data of Högfeldt *et al.*[7] for the Ag^+—H^+ exchange on Wofatit KS and Dowex 50, for which the $\varkappa(\beta)$-curves belong to types I and III respectively. These measurements were made at so low an ionic strength that the variation in the activity of water could be neglected [18] at least to a first approximation. Also the condition $f_{H^+} \cdot f_{Ag^+}^{-1} \approx 1$ is approximately valid[35] and (4b) has been used for calculating $\varkappa$.

The Kroeker equation: This equation was tested by calculating $\log \frac{[Ag]^+_{tot}}{[Ag^+]} = \log \frac{Vc_0}{V\alpha a_0} = q_1$ using the data in Tables 7a and 8a in Ref. 7. Some corresponding values of β and q_1 are given in Table 2. β is the mole fraction of silver in the resin. If the Kroeker equation were valid q_1 should be constant but it is apparent that q_1 decreases with increasing mole fraction. This decrease is systematic and so large that it cannot be due to experimental error, but must be taken as an indication of the inapplicability of the Kroeker equation to the data under consideration. That the Kroeker equation cannot be strictly applicable to either of the two systems is evident from the fact that it must give

Table 1. The relation between the empirical equations and the function $\varkappa(\beta)$.

Author	Equation	Linear plot	$\varkappa(\beta)$	Type	Condition
Kroeker [13]	$\log(Vc_0/Va a_0)=km=q_1$		$\varkappa_{B,A}=\dfrac{(1-q_2\beta)}{q_2(1-\beta)}$ $q_2=s_0/(Va_0(10^{q_1}-1))$	(I) II	$q_2>1$ $q_2<1$
Vageler [15]	$\beta s_0=SVc_0/(Vc_0+k)$	$1/\beta s_0$; $1/Vc_0$	$\varkappa_{B,A}=\dfrac{[q_1-(1+q_2q_3)\beta-q_2\beta^2]}{q_2(1-\beta)(q_3+\beta)}$ $q_1=S/s_0;q_2=s_0/Va_0;q_3=(k-S)/s_0$	I III	$q_1\leq 1+q_2+q_2q_3$ $q_1>1+q_2+q_2q_3$
Weisz [16]	$\beta=Sa/(a+k)$	$1/\beta$; $1/a$	$\varkappa_{B,A}=\dfrac{S-(1+k)\beta}{k(1-\beta)}$	(I) II	$k+1>S$ $k+1<S$
Boedeker [9]	$\beta s_0=k(Vc_0)^p$	$\log\beta s_0$; $\log Vc_0$	$\varkappa_{B,A}=\dfrac{(1+q_1q_2\beta-q_1\beta^{1/p})}{q_1(1-\beta)(\beta^{1/p-1}-q_2)}$ $q_1=s_0{}^{1/p}/Va_0k^{1/p};q_2=k^{1/p}/s_0{}^{1/p-1}$	II III	$p\geq 1$ $p<1$
"Freundlich" [11]	$\beta=ka^p$	$\log\beta$; $\log a$	$\varkappa_{B,A}=\dfrac{k^{1/p}-\beta^{1/p}}{\beta^{1/p-1}(1-\beta)}$	II III (I) (III)	$p\geq 1$ $p<1$ $p\leq 1$ $p>1$
Wiegner-Jenny [17]	$\beta s_0=k\cdot[Va a_0/(Vc_0-Va a_0)]^p$	$\log\beta$; $\log a$	$\varkappa_{B,A}=\dfrac{(1-q_1\beta^{1/p+1})}{q_1(1-\beta)\beta^{1/p}}$; $q_1=\dfrac{s_0{}^{1/p+1}}{Va_0k^{1/p}}$	III (I)	$q_1<1$ $q_1\geq 1$
van Dranen [10]	$\sec\dfrac{\pi}{2}\beta=(\cos\dfrac{\pi}{2}\beta)^{-1}=ka+1$	$\sec\dfrac{\pi}{2}\beta$; a	$\varkappa_{B,A}=\dfrac{\beta(k+1-\sec\frac{\pi}{2}\beta)}{(1-\beta)(\sec\frac{\pi}{2}\beta-1)}$	(I)	
Rothmund-Kornfeld [13-14]	$\beta/(1-\beta)=k[a/(1-a)]^p$	$\log\dfrac{\beta}{1-\beta}$; $\log\dfrac{a}{1-a}$	$\varkappa_{B,A}=k^{1/p}\left(\dfrac{1-\beta}{\beta}\right)^{1/p-1}$	I II	$p<1$ $p>1$

The symbols for the empirical constants are not always the original ones, but are those used in the present work. The terms q_i, $i = 1, 2, 3$ are derived quantities, which are constant under certain experimental conditions. S, k and p are constants, and m is the mass of exchanger. In the last column but one giving the type of $\varkappa(\beta)$-curve obtained the values within paranthesis refer to those curves, which are not valid within the whole concentration range $0 \leq \beta \leq 1$ because of lacking physical meaning.

Table 2. Determination of q_1 in the Kroeker equation.

Wofatit KS		Dowex 50	
$\beta = \frac{[AgR]}{s_0}$	q_1	$\beta = \frac{[AgR]}{s_0}$	q_1
0.002429	0.79	0.002186	0.81
0.01143	0.69	0.01053	0.72
0.05699	0.52	0.05945	0.64
0.1056	0.41	0.1170	0.60
0.2075	0.33	0.1905	0.58
0.3211	0.28	0.2763	0.57
0.4033	0.23	0.3643	0.53
0.5102	0.24	0.4402	0.52
0.6193	0.16	0.5153	0.47
0.7088	0.13	0.6601	0.40
0.7931	0.12	0.8252	0.33
0.9153	0.08	0.9038	0.25

$\varkappa(\beta)$-curves belonging to type II for q_1-values less than 1, whereas the two systems studied belong to types I and III.

Wiegner [19] has tested the Kroeker equation on the ammonium-calcium-permutit system and also obtained decreasing q_1-values with increasing ammonium concentration in the exchanger.

The Vageler equation: The test of this equation was performed as follows. As indicated in Table 1 a plot of $1/\beta\, s_0 = 1/[AgR]$ against $1/Vc_0 = 1/[Ag^+]_{tot}$ would be a straight line if the Vageler equation were applicable. Quite good straight lines were obtained for the two sets of measurements, but the data for Wofatit KS showed a greater spread than those for Dowex 50. The best straight lines were drawn through the experimental points, and the constants S and k were determined. Experimental conditions were such that $a_0 = 100.0$ mC, $V = 0.100$ litre and $s_0 = 1.75$ for Wofatit KS and $s_0 = 3.83$ for Dowex 50. The s_0-values are average values taken from Tables 7a and 8a in Ref. 7. Using these values for a_0, V and s_0 and the values of S and k from the linear plots, the constants q_1, q_2 and q_3 (see Table 1) and the function $\varkappa(\beta)$ were calculated. In Figs. 2 and 3 the full curves give $\varkappa(\beta)$ calculated from the Vageler equation. From these figures it is apparent that the Vageler equation is applicable to a rather large concentration range. Agreement seems to be better for Wofatit KS despite the larger spread in the linear plot. According to Vageler [15], S in his equation should be equal to the saturation capacity s_0. From the linear plots S was found to be 2.0 for Wofatit KS and 10.1 for Dowex 50. These figures are not in agreement with the values of s_0 determined experimentally, especially for Dowex 50. An analogous result was obtained by Weisz [16] for zeolites.

The Weisz equation: The constants S and k (see Table 1) were determined from the plot of $1/\beta$ against $1/\alpha$. This plot yielded a good straight line for Dowex 50, but for Wofatit KS the deviations were greater. From Table 1 it is seen that for $k + 1 > S$ the $\varkappa(\beta)$-curve belongs to type I and for $k + 1 < S$ it belongs to type II. These two conditions were fulfilled for the data on Wofatit KS and Dowex 50 respectively. From Table 1 it is seen that for $\beta = S/(1 + k)$

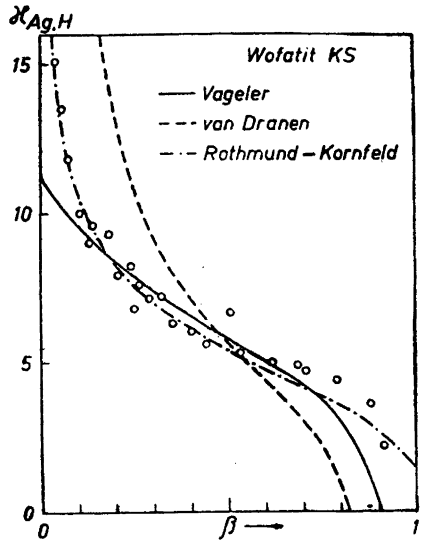

Fig. 2. Comparison between experimental and approximate $\varkappa(\beta)$-curves using the data on Wofatit KS.

○ *Experimental points*
——— $\varkappa(\beta)$ *calculated from the Vageler equation.*
– – – – » » » » *van Dranen* »
— · — · — » » » » *Rothmund-Kornfeld equation.*

$\varkappa(\beta) = 0$. This means that the curve has physical meaning only in the range $0 \leq \beta \leq S/(1 + k)$. For Wofatit KS this value of β is ~ 0.47, a value for which $\varkappa(\beta) = 0$ differs so largely from that for $\beta = 0.47$ in Fig. 2 that the Weisz equation does not appear to be applicable in this case. Although the data on Dowex 50 give a $\varkappa(\beta)$-curve of type III it seems worth-while to test the Weisz equation on the rising part of the curve. In Fig. 3, $\varkappa(\beta)$ is given for Dowex 50 and there is good agreement with the Weisz equation within the interval $0.2 \leq \beta \leq 0.8$. For lower and larger β-values the deviations are large. The occurrence of deviations for small β-values follows from the existence of the minimum in the $\varkappa(\beta)$-curve which cannot be accounted for by the Weisz equation.

Weisz introduced the equation discussed above in his study of various empirical equations [16]. When comparing it with the Vageler equation, he found the latter to give a better numerical agreement. This may perhaps be due to the fact that the Vageler equation is capable of describing curves of both types I and III, while the Weisz equation can only describe part of a curve of type I and curves of type II and should thus have less applicability.

The equation introduced by Weisz can be considered to be a special case of the one used by Wolff [20]:

$$\beta s_0 - k_0 = \frac{S \cdot a}{a + k} \tag{8}$$

where k_0 is a third empirical constant.

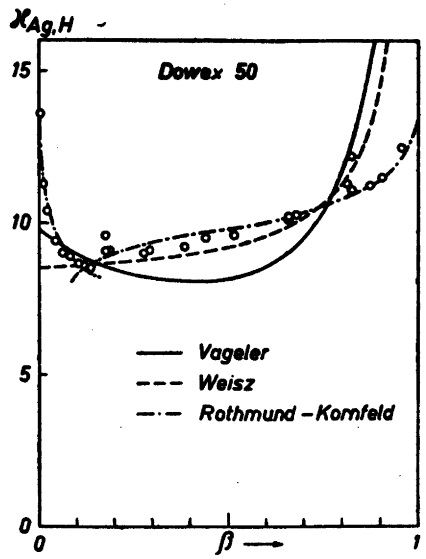

Fig. 3. Comparison between experimental and approximate $\varkappa(\beta)$-curves using the data on Dowex 50.

O *Experimental points.*
——— $\varkappa(\beta)$ *calculated from the Vageler equation.*
– – – – » » » *Weisz* »
– · – · – » » » » *Rothmund-Kornfeld equation.*

When using the Rothmund-Kornfeld equation the two parts of the $\varkappa(\beta)$-curve have been approximated with two different equations, one for the falling and the other for the rising part.

The existence of the constant k_0 means that the equilibrium quotient tends towards infinity as $\beta \rightarrow k_0/s_0$. For a number of cases $\varkappa(\beta)$ increases largely with decreasing β, but in the cases studied right down to the tracer level [21,22] finite and constant limiting values of $\varkappa$ have been obtained. These results imply that k_0 must be very small and the Wolff equation becomes practically equal to the Weisz equation. For such cases the Weisz equation must be preferred even if a better numerical agreement can be obtained from the Wolff equation over a limited concentration range since it contains three empirical constants.

The Boedeker equation: The constants k and p (see Table 1) were first determined from the plot of log βs_0 against log $[Ag^+]_{tot}$ for the two systems. Quite good straight lines were obtained except for the highest values of β, where the experimental points tend towards the log $[Ag^+]_{tot}$-axis. Agreement was better for the data on Dowex 50 than on Wofatit KS, and this can be understood from the fact that the Boedeker equation is applicable to $\varkappa(\beta)$-curves of types II and III only. For this reason the $\varkappa(\beta)$-curve was calculated for Dowex 50 but not for Wofatit KS. The graphical method for finding the constants proved to be too insensitive as it gave a $\varkappa(\beta)$-curve far from the one obtained experimentally. For that reason the method of averages [23] was used for the calculation of k and p. In Fig. 4 the corresponding $\varkappa(\beta)$-curve is plotted. As is seen from this figure agreement is quite good for values of $\beta \lesssim 0.4$. For

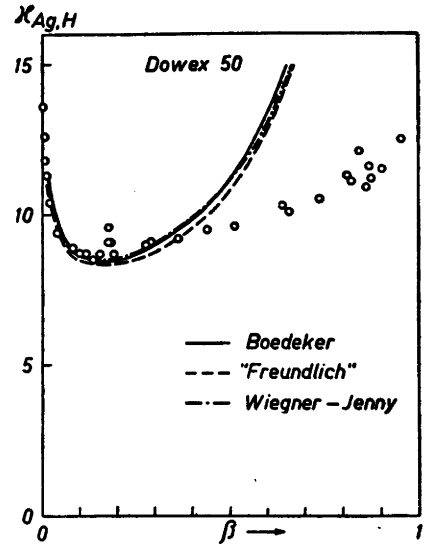

Fig. 4. Comparison between experimental and approximate $\varkappa(\beta)$-curves using the data on Dowex 50.

○ *Experimental points.*
——— $\varkappa(\beta)$ *calculated from the Boedeker equation.*
– – – – » » » *"Freundlich" equation.*
—·—·— » » » *Wiegner-Jenny equation.*

higher values of β the curve tends rapidly towards infinity and deviations from the experimental curve are large. This might be ascribed to the fact that the Boedeker equation (as well as the "Freundlich" and Wiegner-Jenny equations discussed below) is of an exponential type and does not indicate the existence of an upper limit for the amount to be exchanged.

The "Freundlich" equation: The constants k and p (see Table 1) were determined from the plot of log β against log α for Dowex 50. The $\varkappa(\beta)$-curve has been plotted in Fig. 4. The close agreement with the $\varkappa(\beta)$-curve obtained from the Boedeker equation is to be noted and the same arguments are applicable to them both. When testing the equation with the data on Wofatit KS k proved to be > 1 and for $k > 1$ the "Freundlich" equation is inapplicable to a $\varkappa(\beta)$-curve of type I and the $\varkappa(\beta)$-curve was not calculated.

Freundlich's name is given within quotation marks, because van Bemmelen [24] (as is pointed out by McBain [25]) was the originator of the equation, although it has become associated with Freundlich's name.

The Wiegner-Jenny equation: This is a modification of the "Freundlich" equation put forward in order to indicate that the ion exchange equilibrium is independent of dilution for exchange between ions of equal valence. The quantities p and q_1 (q_1 constant under the experimental conditions) were determined from the plot of log α against log β. That this plot should give a straight line is seen by the following argument:

The Wiegner-Jenny equation:

$$\beta s_0 = k \cdot \left(\frac{V a a_0}{V c_0 - V a a_0} \right)^p$$

and the requirement of conservation of mass (6) give:

$$a = q_1 \beta^{1/p+1} \qquad q_1 = \frac{s_0^{1/p+1}}{V a_0 k^{1/p}} \tag{9}$$

From (9) it is seen that for the case of constant V and a_0 the Wiegner-Jenny equation becomes a special case of the "Freundlich" equation. It is also seen that by plotting log a against log β a straight line should be obtained giving the values of $1/p + 1$ and q_1. $\varkappa(\beta)$ calculated from the Wiegner-Jenny equation, is plotted in Fig. 4 for the Ag^+—H^+ exchange on Dowex 50. The measurements on Wofatit KS were not used for the same reasons as for the Boedeker and "Freundlich" equations. The $\varkappa(\beta)$-curves in Fig. 4 from the "Freundlich" and Wiegner-Jenny equations should have coincided. The observed differences are due to small differences in the constants ($\sim$ 1 %) depending upon the limited accuracy in their determination from the linear plot and shows the sensitivity of the $\varkappa(\beta)$-curve to variations in the constants.

The van Dranen equation: Recently van Dranen [10] has proposed the equation given in Table 1 containing only one parameter and having an upper limit equal to the saturation capacity. The equation was tested by calculating $k = (\sec \frac{\pi}{2} \beta - 1)/a$ for the two sets of measurements. No constant values were obtained as is shown in Table 3, where some of the results for Wofatit KS are given. The variation in k is large and the results show that this equation is not applicable to the data under consideration. An analogous result was obtained for Dowex 50, but this can be understood from the inapplicability of the van Dranen equation to a $\varkappa(\beta)$-curve of type III.

The form of a $\varkappa(\beta)$-curve calculated from the van Dranen equation, has been obtained by using the value $k = 2.74$ and the resulting curve is given in Fig. 2.

Table 3. Determination of $k = (\sec \frac{\pi}{2} \beta - 1)/a$ *in the van Dranen equation using the data on Wofatit KS.*

$\beta = \frac{[AgR]}{s_0}$	$(\sec \frac{\pi}{2} \beta - 1)/a$	$\beta = \frac{[AgR]}{s_0}$	$(\sec \frac{\pi}{2} \beta - 1)/a$
0.002429	0	0.2075	1.74
0.006871	0.323	0.2515	1.78
0.009246	0.254	0.3211	2.31
0.01336	0.298	0.4033	2.36
0.01946	0.362	0.5102	3.23
0.02810	0.537	0.6193	3.18
0.04129	0.740	0.6858	3.56
0.05699	0.817	0.7088	3.73
0.07582	1.02	0.7931	4.60
0.1056	1.19	0.8825	6.58
0.1419	1.51	0.9153	7.81

The Rothmund-Kornfeld equation: Monovalent ions. In Fig. 2 the curve denoted by —·—·— gives $\varkappa(\beta)$ obtained by applying the Rothmund-Kornfeld equation (for brevity called the R—K equation in the following) to the data on Wofatit KS. k and p were determined from the plot of $\log \frac{\beta}{1-\beta}$ against $\log \frac{\alpha}{1-\alpha}$ which yielded a good straight line. Agreement is seen to be good over the whole concentration range. That the R—K equation (types I or II) is not applicable to the data on Dowex 50 (type III) can be seen as a small non-linearity of the insensitive double-logarithmic plot. The R—K equation can, however, be applied to curves of type III by proceeding as follows. The rising part of the curve is approximated by a R—K equation with $p > 1$ and the falling part by a R—K equation with $p < 1$. The four parameters k_1, p_1 and k_2, p_2 can all be determined from the double-logarithmic plot because, in the case of a curve of type III, the double-logarithmic plot can be approximated by two straight lines intersecting at the point corresponding to the minimum or maximum.

In Fig. 3 the curve denoted by —·—· gives $\varkappa(\beta)$ obtained using two different R—K equations intersecting at $\beta = 0.12$. Agreement is seen to be quite good.

Multivalent ions: The R—K equation seems to be the most useful one, as it can be applied to all three types of $\varkappa(\beta)$-curves and gives good numerical agreement for the two experimental studies considered here. For that reason, the discussion concerning multivalent ions will be limited to this equation. When considering multivalent ions, two approaches are possible as discussed in a previous paper[18]: "choice" (E) ("equivalents") and "choice" (M) ("moles").

Choice (E) gives the following ion exchange reaction:

$$z_A B^{z_B+} + z_A z_B (A_{1/z_A})R \rightleftharpoons z_B A^{z_A+} + z_A z_B (B_{1/z_B})R \qquad (10)$$

The equilibrium quotient $\varkappa_{B,A}$ for reaction (10) is defined by:

$$\varkappa_{B,A} = \frac{\{A^{z_A+}\}^{z_B}}{\{B^{z_B+}\}^{z_A}} \left(\frac{[(B_{1/z_B})R]}{[(A_{1/z_A})R]}\right)^{z_A z_B} = \frac{\{A^{z_A+}\}^{z_B}}{\{B^{z_B+}\}^{z_A}} \left(\frac{\beta}{1-\beta}\right)^{z_A z_B} \qquad (11)$$

if the concentrations in the exchanger are expressed by the equivalent fractions:

$$\beta = \frac{[(B_{1/z_B})R]}{s_0} \text{ and } 1-\beta = \frac{[(A_{1/z_A})R]}{s_0} \qquad (12)$$

The R—K equation then takes the following form:

$$\left(\frac{[(B_{1/z_B})R]}{[(A_{1/z_A})R]}\right)^{z_A z_B} = k \left(\frac{\{B^{z_B+}\}^{z_A}}{\{A^{z_A+}\}^{z_B}}\right)^{p} \qquad (13)$$

where k and p are empirical constants.

From (11) and (13) the following expression for $\varkappa(\beta)$ can be deduced:

$$\varkappa_{B,A} = k^{1/p} \left(\frac{(1-\beta)}{\beta}\right)^{z_A z_B (1/p - 1)} \qquad (14)$$

$\varkappa_{B,A}$ belongs to type I if $p < 1$
» » » » II » $p > 1$

Choice (M) gives the following ion exchange reaction:

$$z_A B^{z_B+} + z_B AR_{z_A} \rightleftharpoons z_B A^{z_A+} + z_A BR_{z_B} \tag{15}$$

and the equilibrium quotient for reaction (15) is defined by:

$$\lambda_{B,A} = \frac{\{A^{z_A+}\}^{z_B} [BR_{z_B}]^{z_A}}{\{B^{z_B+}\}^{z_B} [AR_{z_A}]^{z_B}} \tag{16}$$

The R—K equation now gives:

$$\frac{[BR_{z_B}]^{z_A}}{[AR_{z_A}]^{z_B}} = k \left(\frac{\{B^{z_B+}\}^{z_A}}{\{A^{z_A+}\}^{z_B}} \right)^p \tag{17}$$

From (16) and (17):

$$\lambda_{B,A} = k^{1/p} \frac{(1-X_B)^{z_B(1/p-1)}}{X_B^{z_A(1/p-1)}} \tag{18}$$

where the mole fractions X_B and $(1-X_B)$ are defined by:

$$X_B = \frac{[BR_{z_B}]}{[AR_{z_A}] + [BR_{z_B}]} \text{ and } 1-X_B = \frac{[AR_{z_A}]}{[AR_{z_A}] + [BR_{z_B}]} \tag{19}$$

From (18) it is seen:

For $p < 1$ $\lambda_{B,A}$ belongs to type I
» $p > 1$ $\lambda_{B,A}$ » » » II

For these two cases too, curves of type III can be approximated using the extension discussed for monovalent ions.

The thermodynamic equilibrium constant: The R—K equation can also be used for obtaining an approximate value for the thermodynamic equilibrium constant for the ion exchange process. This quantity is of value when discussing ion exchange affinities.

Argersinger *et al.*[26,27] as well as Högfeldt *et al.*[18,28–30] have shown that the thermodynamic equilibrium constant ($K_{B,A}$) can be obtained by graphical integration of the log $\varkappa_{B,A}(\beta)$-curve. For the calculation of $K_{B,A}$ the following expression can be used:

$$\ln K_{B,A} = \int_0^1 \ln \varkappa_{B,A}(\beta) d\beta \tag{20}$$

When taking the variation in the activity of water into consideration a more complex expression may be obtained [27,31].

Inserting the expression for $\varkappa_{B,A}$ from the R—K equation in (20) and integrating the following expression for $K_{B,A}$ is obtained:

$$K_{B,A} = k^{1/p} \tag{21}$$

This quantity, $k^{1/p}$, can be obtained directly from the double-logarithmic plot, where the intercept on the abscissa gives $-\log k^{1/p}$. For multivalent ions only choice (E) gives the same result as that given by (21).

Equation (20) can also be applied to the other empirical equations discussed in this paper, but the R—K equation gives the simplest and most straight-forward method for getting an approximate value for $K_{B,A}$.

In connection with equation (21) it may be noted that it has been proposed [32] that the $\varkappa$-value for $\beta = 0.5$ is a good measure for the relative affinity of the ions for the exchanger. For a system, which can be approximated by the R—K equation, it is found from equation (21) that:

$$\varkappa_{B,A(\beta=0.5)} = K_{B,A} \tag{22}$$

For systems with a maximum or minimum in the $\varkappa(\beta)$-plot, the following approximate expression obtained from (20) and the R—K equation may be useful:

$$\log K_{B,A} \approx \beta_m \log k_1^{1/p_1} + (1-\beta_m) \log k_2^{1/p_2} \tag{23}$$

where β_m is the β-value for the maximum or minimum and k_1, p_1 and k_2, p_2 are the parameters for the two parts of the $\varkappa(\beta)$-curve.

In order to test the usefulness of the different approximate expressions for $K_{B,A}$, $K_{Ag,H}$ was calculated for the two studies of the Ag^+—H^+ exchange on Wofatit KS and Dowex 50 and compared with $\varkappa_{Ag,H(\beta=0.5)}$ and $K_{Ag,H}$-values obtained by graphical integration [18]. The results are given in Table 4.

Table 4. A comparison between approximate and integrated $K_{Ag,H}$-values.

Exchanger	k_1	p_1	k_2	p_2	β_m	$\varkappa_{Ag,H}$ $\beta = 0.5$	$K_{Ag,H}$ from (21) or (23)	$K_{Ag,H}$ integrated
Wofatits KS	3.59	0.75	—	—	—	5.4	5.50	5.16
Dowex 50	5.01	0.89	11.2	1.04	0.14	9.6	9.42	9.90

From Table 4 it is seen that, for the two cases studied, the approximation from the R—K equation gives results in agreement with the integration within $\pm$ 10 % — an accuracy which may often be sufficient. It is also seen that the simpler method using $\varkappa_{B,A(\beta=0.5)}$ is also of sufficient accuracy and for a first approximation it may be useful in a large number of cases.

Recently the authors attention has been called to a paper by Yamabe and Sato [33] which describes the use of an empirical equation which in our notation becomes:

$$\log \frac{\beta}{\alpha} = p \log \frac{(1-\beta)}{(1-\alpha)} + \log k \tag{24}$$

where p and k are empirical constants. A plot of $\log \frac{\beta}{\alpha}$ against $\log \frac{(1-\beta)}{(1-\alpha)}$ should be a straight line. In their test of the equation to exchange between mono- and divalent ions the authors claim that better agreement was obtained by plotting $\log \frac{\beta s_0}{V a a_0}$ against $\log \frac{s_0(1-\beta)}{V a_0(1-\alpha)}$. In testing this equation with the data of Högfeldt *et al.*[7] V, a_0 and s_0

are practically constant so that the test can equally well be performed on (24) as on the alternative. In order to calculate $\varkappa(\beta)$ from (24) it is convenient to express $\beta(\varkappa)$ as:

$$\beta = \frac{\varkappa(k^{1/(p-1)} - \varkappa^{1/(p-1)})}{k^{1/(p-1)}(\varkappa - 1)} \tag{25}$$

From (25) it can be deduced that:

$\left.\begin{matrix} p>1 \\ k>1 \end{matrix}\right\}$ type I $\left.\begin{matrix} p>1 \\ k<1 \end{matrix}\right\}$ type II $\left.\begin{matrix} p<1 \\ k>1 \end{matrix}\right\}$ type II $\left.\begin{matrix} p<1 \\ k<1 \end{matrix}\right\}$ type I

and thus (24) can be used on $\varkappa(\beta)$-curves of types I and II.

The equation was tested with the data on Wofatit KS but no straight line was obtained. It did not seem worth-while calculating $\varkappa(\beta)$ as the insensitive double-logarithmic plot showed large deviations from linearity. As Yamabe [34] has obtained good agreement with experimental data for higher concentrations than 0.1 C the present results suggest that his equation may be less useful at lower concentrations.

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DISCUSSION

From Figs. 2—4 it is seen that it always seems possible to find an empirical equation applicable to a limited concentration range, whereas it may be hard to find one applicable to the whole concentration range. An indispensible condition is that the empirical equation can give a $\varkappa(\beta)$-curve of the same type, I—III, (see Fig. 1 and Table 1) as the experimental one.

The linear plots (which are often logarithmic) used to determine the constants in the empirical equations seem to be too insensitive to test agreement between the empirical equation and the experimental data. They may, however, be used for smoothing out experimental data.

For the two sets of measurements on which the empirical equations have been tested the best agreement is found with the R—K equation. Although the R—K equation is only applicable to $\varkappa(\beta)$-curves of types I and II it is relatively easy to extend its usefulness to all types. None of the other equations seems to offer this extension in such a simple manner. However, from a test on only two experimental $\varkappa(\beta)$-curves, no general deductions can be made about its accuracy in other cases.

The present conclusion, that the R—K equation is the most useful one, is in contradiction with the results of Weisz [16], who found that the Vageler equation gave better numerical agreement with experimental data on zeolites than the equations of "Freundlich", Rothmund-Kornfeld, Wiegner-Jenny and himself. Weisz tested the empirical equations on several systems containing both monovalent and multivalent ions, but the concentration ranges were not as broad as in the present investigation. However, Weisz found, as in this investigation, that the exponential curves ("Freundlich", Wiegner-Jenny) did not give as good an agreement as curves of the hyperbolic type (Vageler, Weisz). This may be due to the fact that the hyperbolic curves (in agreement with experience) have an upper limit for the exchange not shown by the exponential ones.

Future work: There are a number of theoretical approaches in the literature giving a functional connection between α and β. In a following paper they will be discussed in an analogous manner.

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Short Communications

Über die Eigenschaften des Lösungsmittels, die die Kinetik der alkalischen Esterhydrolyse bestimmen. I. Der Zusammenhang zwischen Aktivierungswärme und Protonenübergangswärme

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Die Geschwindigkeit der durch Alkalien hervorgerufenen Esterhydrolyse hängt viel vom Lösungsmittel ab. Es fragt sich, in welcher direkten Weise die katalytische Aktivität des alkalihaltigen Lösungsmittels gemessen werden kann. Die Antwort lautet, dass die Messung mittels einem dem Aktivierungsprozess ähnlichen Prozess ausführbar ist.

Man ist der Ansicht, dass der Aktivierungsprozess der alkalischen Esterhydrolyse von der Bildung des Additionsproduktes EOH^- aus Estermolekül E und Hydroxylion bedingt ist [1]. Dieser Fall lässt sich, abweichend vom Üblichen, durch die Gleichung $E + H_2O \rightleftharpoons EOH^- + H^+$ beschreiben. Die Gleichung für die Autoprotolyse des Lösungsmittels HL ist $[H^+] = K[HL]/[L^-]$. Diese beiden Gleichungen ergeben (1) $[EOH^-]/[E][H_2O] = K^*[L]/[HL]$. Die Konstante K^* des Aktivierungsgleichgewichts hängt natürlich von der Art des Lösungsmittels HL ab.

Die Form der Gleichung (1) unterscheidet sich von der der üblichen Säure-Basen-Gleichgewichtsgleichung (2) $[A^-]/[HA] = K[L^-]/[HL]$ nur darin, dass erstere Gleichung 3, letztere 2 neutrale Moleküle enthält. Experimentell ist belegt, dass die relative Säurestärke der Protonenspender nicht sehr viel vom Lösungsmittel abhängt [2]. Dies bedeutet, dass der Einfluss des Lösungsmittels auf das Verhältnis der Aktivitätskoeffizienten der Säure und ihres Anions bei den verschiedenen Säuren gleichgross ist. Es ist anzunehmen, dass dies auch für Gleichung (1) gültig ist, wobei $[E]\,[H_2O]$ sich also wie [HA] verhält.

Auf Grund des Vorstehenden sollte also das Verhältnis K^*/K der Gleichgewichtskonstanten der Gleichungen (1) und (2) unabhängig vom Lösungsmittel HL sein, und da das Vorstehende nicht auf eine bestimmte Temperatur beschränkt ist, so muss der Einfluss der Temperatur auf das Verhältnis K^*/K unabhängig vom Lösungsmittel sein. Durch Anwendung der van't Hoffschen Gleichung erhält man $RT^2(d\ln K^* - d\ln K)/dT = \triangle H^* - H =$ unabhängig vom Lösungsmittel. $\triangle H^*$ ist die Aktivierungswärme der Hydrolyse im Lösungsmittel HL, $\triangle H$ ist die Wärme der Protonenübergangsreaktion $HA + L^- \rightarrow A^- + HL$. Bezeichnet man die dem Lösungsmittel L_1 entsprechenden Wärmen mit $\triangle H_1^*$ und $\triangle H_1$, die dem Lösungsmittel L_2 entsprechenden mit $\triangle H_2^*$ und $\triangle H_2$, so ist $\triangle H_1^* - \triangle H_2^* = \triangle H_1 - \triangle H_2$. Die »Neutralisierungswärme» kommt somit der Aktivierungswärme zugute.

Die Protonenübergangswärmen wurden kalorimetrisch gemessen, 25° C, unter Verwendung von Phenol und Essigsäure, in einigen Fällen auch Ameisensäure, Bernsteinsäure und Benzoesäure als Protonenspender. Bei diesen Substanzen war die Protonenübergangswärme in ziemlich gleicher Weise vom Lösungsmittel abhängig [3].

Aus den in der Literatur beschriebenen Versuchen über die alkalische Hydrolyse der Ester geht hervor, dass der Einfluss des Lösungsmittels bei verschiedenen Estern von ziemlich gleicher Art ist, wie es oft der Fall ist bei Reaktionen von gleichem Typus. Die vom Verfasser ausgeführten Versuche bekräftigen diese Auffassung. (Wegen der Umesterung lässt sich in viel Alkohol enthaltenden Lösungsmitteln nur die Hydrolysengeschwindigkeit des Esters des betreffenden Alkohols messen.) Die in

den Versuchen verwendeten Lösungsmittel, neben rein wässrigen Lösungen, und Ester, sind die folgenden:

Die Numerierung der Lösungsmittel in den Figuren ist dieselbe wie im vorstehenden Verzeichnis.

	Lösungsmittel	Ester
1.	Wasser — Phenol	Äthylacetat, *n*-Propylacetat
2.	» — Rohrzucker	Methyl-, Äthyl- und Propylacetat
3.	» — Glyzerin	Äthylacetat
4.	» — Äthylenglykol	»
5.	» — Methanol	Methylacetat und -benzoat
6.	» — Aceton	Äthylacetat
7.	» — Äthanol	»
8.	» — *n*-Propanol	» , *n*-Propylacetat, Äthylbenzoat
9.	» — *i*-Propanol	» , *i*-Propylacetat
10.	» — *tert.* Butanol	» , Methylacetat, Äthylbenzoat

Als Katalysator diente NaOH. Die Ergebnisse der Versuche sind in den Figuren 1—3 wiedergegeben, von denen Figur 1 die Abhängigkeit der Aktivierungswärme vom Lösungsmittel, Figuren 2 und 3 die entsprechende Abhängigkeit der Protonenübergangswärme darstellen. Falls die Aktivierungswärmen unter Verwendung verschiedener Ester im gleichen Lösungsmittel bestimmt worden sind, so entsprechen deutlichkeitshalber die Punkte der Figur 1 dem Mittelwert. Als Protonenspender dienten Phenol (Fig. 2) und Essigsäure (Fig. 3). Der Deutlichkeit halber zeigen die Figuren nur die Änderungen $\triangle H - \triangle H_0$ dieser Wärmen in Bezug auf den in wässriger Lösung vorliegenden Wert $\triangle H_0$.

Die Gleichartigkeit der Figur 1 mit den Figuren 2 und 3 zeigt auf das Deutlichste, dass die beschriebene Protonenübergangswärme in die Aktivierungswärme der alkalischen Hydrolyse der Ester eingeht. Die Übereinstimmung kann natürlich nicht völlig quantitativ sein, schon weil der zweite Energiefaktor, der die relative Säurestärke mitbestimmt, die Protonenübergangswärme, offenbar zu einem gewissen Grade vom Lösungsmittel abhängt, wie auch die relative Säurestärke selbst (Figuren 2 und 3). Aber obwohl die benutzten Protonenspender, Phenol und Essigsäure, auf Grund ihrer allgemeinen chemischen Eigenschaften zu verschiedenen Substanzgruppen gehören, so sind die

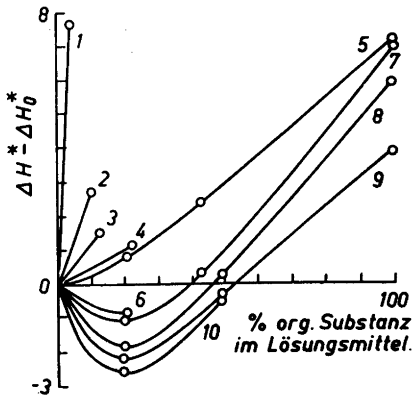


Fig. 1. Abhängigkeit der Aktivierungswärme vom Lösungsmittel.

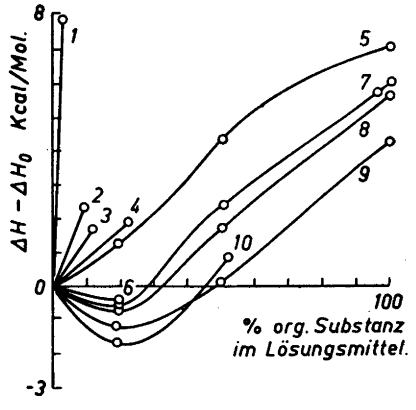


Fig. 2. Abhängigkeit der Protonenübergangswärme vom Lösungsmittel. Protonenspender: Phenol.

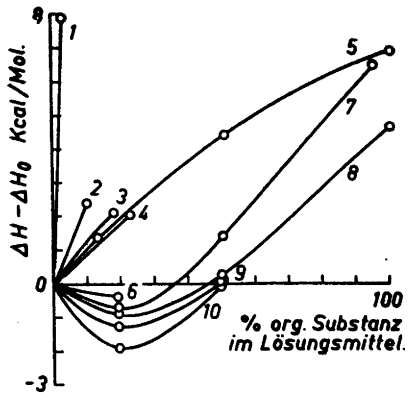


Fig. 3. Abhängigkeit der Protonenübergangswärme vom Lösungsmittel. Protonenspender: Essigsäure.

Unterschiede lange nicht so gross, dass sie das Gesamtbild veränderten.

Es ist auffallend, dass der Protonenübergang an das Alkoxyl-Ion in alkoholischer Lösung viel weniger Wärme befreit als derjenige an das Hydroxyl-Ion in wässriger Lösung.

1. Day, J. N. E. und Ingold, C. K. *Trans. Faraday Soc.* **37** (1941) 686; Bender, M. L. *J. Am. Chem. Soc.* **73** (1951) 1626.
2. Siehe Literaturverzeichnis: Dippy, J. F. J. *J. Chem. Soc.* **1941** 550; *Chem. Revs.* **25** (1939) 151.
3. Siehe auch Wolfenden, J. H., Jackson, W. und Hartley, H. B. *J. Phys. Chem.* **31** (1927) 850.

Eingegangen am 24. November 1954.

Low-molecular Carbohydrates in Algae

VII *. Investigation of *Fucus spiralis* and *Desmarestia aculeata*

HANS BOUVENG and BENGT LINDBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

In Parts I [1], IV [2] and V [3] of this series, studies on the brown algae *Fucus vesiculosus*, *Pelvetia canaliculata* and *Laminaria cloustoni* were reported. These algae all contained mannitol, 1-mannitol β-glucoside and 1,6-mannitol di-(β-glucoside). 1-Mannitol acetate was isolated from *F. vesiculosus* and *L. cloustoni*, volemitol and its mono- and di-glucosides from *P. canaliculata* and a substance, laminitol, later proved to be a C-methyl inositol (Part VI) from *L. cloustoni*. It was therefore of interest to investigate other brown algae to see if some of these substances were characteristic for the whole group, and in the present communication the investigation of *Fucus spiralis* and *Desmarestia aculeata* is reported. Mannitol, 1-mannitol β-glucoside and laminitol were isolated from both and the presence of 1,6-mannitol diglucoside was demonstrated by paper chromatography. It is most probable that laminitol was overlooked in *F. vesiculosus* and *P. canaliculata* and that this substance, as well as mannitol and its glucosides, is characteristic for brown algae in general. 1-Mannitol acetate was isolated from *F. spiralis* but not from *D. aculeata* and it seems significant that this substance has been obtained in a good yield only from the *Fucus* species. Volemitol was not found in either of the two algae now investigated. It has thus so far only been found in *P. canaliculata* but it is intended to investigate its possible occurrence in other brown algae.

The algae, *Fucus spiralis* (740 g) and *Desmarestia aculeata* (240 g) were kindly supplied by Institutt for Tang- og Tareforskning, Trondheim. They were extracted and the extracts worked up as described in previous communications [3]. After fractionation on carbon columns and, when necessary, further separation on thick filter paper, the following substances were isolated:

Fucus spiralis. Laminitol, 0.18 g (0.025 %). M. p. 245–247° (slight decomp.).

Mannitol, 23 g (3.1 %). M. p. 160–162°.

1-Mannitol acetate, 0.70 g (0.1 %). M. p. 121–122°.

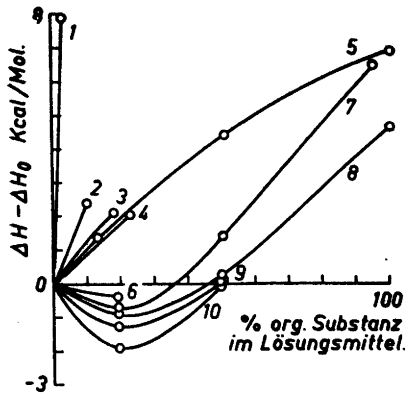
1-Mannitol β-glucoside, 0.30 g (0.04 %). M. p. 141–142°.

1,6-Mannitol di-(β-glucoside), about 0.03 g (0.004 %). Amorphous, chromatographically indistinguishable from authentic material.

Desmarestia aculeata. The crude laminitol was purified as acetate. Yield 20 mg (0.004 %, calculated as laminitol). M. p. 147–148°.

Mannitol, 13.5 g (5.6 %). M. p. 162–163°.

* Part VI, *Acta Chem. Scand.* **8** (1954) 1875.



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* Part VI, *Acta Chem. Scand.* **8** (1954) 1875.

1-Mannitol β-glucoside 0.11 g (0.045 %). M. p. 138–139°.

1,6-Mannitol di-(β-glucoside), about 5 mg (0.002 %). Amorphous, chromatographically indistinguishable from authentic material.

The melting points of all the crystalline compounds were undepressed on admixture with authentic specimens.

Acknowledgement. The authors are indebted to *Statens Naturvetenskapliga Forskningsråd* for financial support and to Ing. J. Dulny for skilful assistance.

1. Lindberg, B. *Acta Chem. Scand.* **7** (1953) 1119.
2. Lindberg, B. and Paju, J. *Acta Chem. Scand.* **8** (1954) 817.
3. Lindberg, B. and McPherson, J. *Acta Chem. Scand.* **8** (1954) 1547.

Received November 26, 1954.

Low-molecular Carbohydrates in Algae

VIII *. Investigation of Two Green Algae

BENGT LINDBERG

Organisk-kemiska Institutionen, Kungl. Tekniska Högskolan, Stockholm, Sweden

Two green algae, one marine, *Enteromorpha compressa*, and one fresh water alga, *Chlorella*, strain Tx 14–10, have been investigated, using the same technique previously applied to brown algae (Part V [1]).

Sucrose was isolated in good yield from both algae and evidence for the occurrence of *meso*-inositol in the two algae was also obtained. A small amount of mannitol was isolated from *E. compressa*, but this might have come from contaminating brown algae. From the *Chlorella* maltose and maltotriose were isolated. These substances certainly have some connection with starch, either as precursors in its biosynthesis or as products of a post-mortal, enzymatic hydrolysis. In addition, the presence of several unidentified substances, occurring in small amounts, was demonstrated by paper chromatography.

Enteromorpha compressa (250 g), kindly supplied by Marinbotaniska Institutionen, Göteborg, was extracted and worked up as previously described for the brown algae [1]. The carbohydrate fraction was separated on a carbon column, using the gradient elution technique. Mannitol (90 mg), m. p. 158–162° and sucrose (1.4 g), m. p. 178–179° were isolated, and the presence of *meso*-inositol demonstrated by paper chromatography.

Chlorella, strain Tx 14–10, (160 g), kindly supplied by Docent L. E. Enebo, Kungl. Tekniska Högskolan, Stockholm, was refluxed for 4 hours with 75 % ethanol (2 000 ml), the extract separated by centrifugation and the residue re-extracted and treated in the same manner. The combined extracts were worked up as above, separated on a carbon column and further fractionated on thick filter paper. The following substances were isolated:

meso-Inositol (0.22 g), m. p. 216–217°. Acetate, m. p. 209–211°. Sucrose (3.8 g), m. p. 182–183°.

Maltose (80 mg) was isolated by further separation of a fraction from the carbon column on thick filter paper. It was amorphous and not quite pure, $[\alpha]_D^{20} + 110°$ (c, 2.0 in water), but was chromatographically indistinguishable from authentic maltose and on hydrolysis yielded glucose only.

Maltotriose (270 mg) was isolated from another fraction in the same manner. It was also amorphous and not quite pure, $[\alpha]_D^{20} + 139°$ (c, 2.0 in water), but chromatographically indistinguishable from authentic maltotriose and on partial hydrolysis yielded glucose and maltose (identified by paper chromatography).

The melting points of all the crystalline compounds were undepressed on admixture with authentic materials.

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1. Lindberg, B. and McPherson, J. *Acta Chem. Scand.* **8** (1954) 1547.

Received November 26, 1954.

* Part VII, *Acta Chem. Scand.* **9** (1955) 168.

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1. Lindberg, B. and McPherson, J. *Acta Chem. Scand.* **8** (1954) 1547.

Received November 26, 1954.

* Part VII, *Acta Chem. Scand.* **9** (1955) 168.

4-Hydroxy-Piperidine-2-Carboxylic Acid in Green Plants

ARTTURI I. VIRTANEN and SIGRID KARI

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

We recently isolated a new piperidine-carboxylic acid from *Rhapis flabelliformis* and identified it as 5-hydroxy-piperidine-2-carboxylic acid [1]. By paper chromatography this substance was found also in *Acacia* species. In this case, however, also another spot in connection with the spot of 5-hydroxy-piperidine-2-carboxylic acid could be seen on the paper chromatogram (Fig. 1). The colour produced with ninhydrin of this spot was at first yellow but turned after heating gradually sky blue, while the spot given by the 5-hydroxy acid was blue tinged with violet. Without this difference in colour it had been difficult at first to observe that two different substances together formed the large spot.

We have now isolated this other amino acid from a 70 % alcohol extract of *Acacia pentadena*. The solution of 655 g of fresh plants (= 346 g dry material, 764 mg soluble N) was first run through an Amberlite IR-120 column (1.5 × 43 cm, 80 ml resin). The amino acids remained in the column and were eluted with 1 *N* ammonia whereby all amino acids came out in fractions Nos. 4—19 of 17 ml each. Ammonia was evaporated *in vacuo* from these fractions. The syrupy residue was dissolved in 10 ml of 1.5 *N* HCl and the solution obtained was passed into a Dowex 50 column (4 × 64 cm, 800 ml resin). The resin had previously been treated with 1.5 *N* HCl. The amino acids were eluted with 1.5 *N* HCl. 400 fractions of 9 ml were collected. The unknown amino acid came out in fractions Nos. 201—260. Fractions 201—220 contained besides α-amino acids (serine, threonine and asparagine) only the unknown amino acid, fractions 221—260 also 5-hydroxy-piperidine-2-carboxylic acid. Fractions 201—220 were therefore used for the isolation of the unknown amino acid. In order to destroy the α-amino acids they were deaminated with nitrogen oxides. The solution was then passed through an Amberlite IR-120 column and the amino acid in question, which was not deaminated, was eluted with ammonia. After evaporation to a small volume the unknown amino acid crystallized in fine flakes. The yield was 180 mg corresponding to about 18 mg N (2.4 % of the soluble N in *Acacia*). When the amount of this amino acid in the mother liquid and in fractions 221—260 are taken into account about 2.7 % of the soluble N in *Acacia* belongs to the unknown amino acid. Analysis of the recrystallized substance; Found: C 49.25; H 7.67; N (Dumas) 9.63. Calc. for hydroxy-piperidine-carboxylic acid $C_6H_{11}O_3N$: C 49.65; H 7.59; N 9.66. M. p. determination: turned brown at 250° C, decomposed at 270° C.

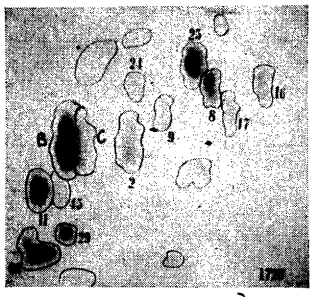


Fig. 1. Two-dimensional paper chromatogram (butanol-acetic acid + phenol-NH_3) from a 70 % alcohol extract of Acacia pentadena. 2 = ala, 8 = ser, 9 = threo, 11 = pro, 16 = asp, 17 = glu, 24 = glu-NH_2, 25 = asp-NH_2, 29 = γ-aminobut. acid, 45 = ethanolamine, 60 = piperidine-2-carboxylic acid (pipecolic acid), B = unknown amino acid, C = 5-hydroxy-piperidine-2-carboxylic acid.

This amino acid could be clearly distinguished from 5-hydroxy-2-piperidine-carboxylic acid by paper chromatography, because it moved somewhat faster in phenol. R_F-values (Whatman No. 4 paper):

	Phenol-NH_3	Butanol-acetic acid
5-hydroxy-piperidine-2-carboxylic acid	0.783	0.254
4-hydroxy-piperidine-2-carboxylic acid (unknown amino acid)	0.826	0.232
α-alanine	0.609	0.247

The new amino acid did not reduce alkaline permanganate solution. Upon reduction with 66 % HI (*d* 1.96) and red phosphorus at 140° C piperidine-2-carboxylic acid (pipecolic acid) was formed (Fig. 2).

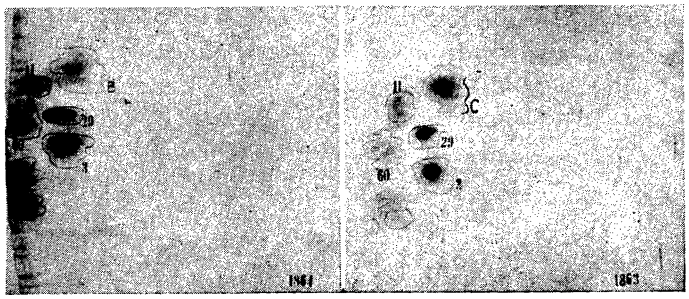


Fig. 2. Products formed from unknown amino acid B and 5-hydroxypiperidine-2-carboxylic acid C after reduction with HI and P. B = unknown amino acid which partly remained unchanged, C = 5-hydroxy - piperidine - 2-carboxylic acid which partly remained unchanged, 60 = pipecolic acid formed from B and C; amino acids 3, 11, and 29 added for comparison. Two lower spots on the chromatograms are unknown, probably I containing substances.

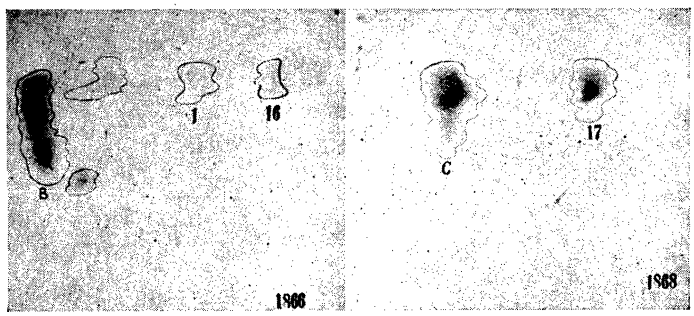
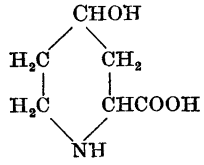


Fig. 3. Products formed from unknown amino acid B and 5-hydroxypiperidine-2-carboxylic acid by oxidation with $KMnO_4$ in H_2SO_4-solution. B = unknown amino acid which partly remained unchanged, C = 5-hydroxypiperidine-2-carboxylic acid which partly remained unchanged, 16 = asp, 17 = glu, 1 = gly.

Accordingly, some hydroxy-piperidine-2-carboxylic acid was in question, having its OH group in another position than 5. On oxidation with potassium permanganate in sulphuric acid solution the substance gave, in addition to two substances not closer investigated, aspartic acid and a smaller amount of glycine, but no glutamic acid (Fig. 3). In parallel experiments our 5-hydroxy-piperidine-2-carboxylic acid gave only glutamic acid. Accordingly the unknown amino acid is 4-hydroxypiperidine-2-carboxylic acid.



In our previous paper we advanced the hypothesis that 5-hydroxy-piperidine-2-carboxylic acid could be formed from δ-hydroxylysine. Correspondingly 4-hydroxy acid could be formed from γ-hydroxylysine. This acid has not yet been found in nature.

In addition to *Acacia pentadena*, *Acacia retinoides* and *Strelitzia reginae* contained both 4- and 5-hydroxy-piperidine-2-carboxylic acid. *Albizzia lophantha* again contained only 4-hydroxypiperidine-2-carboxylic acid.

We wish to thank Professor A. Kalela for the plant material obtained from the University Botanical Gardens.

1. Virtanen, A. I. and Kari, S. *Acta Chem. Scand.* **8** (1954) 1290.

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"Dimethyldioxan" Obtained from Propylene Glycol and Sulphuric Acid Identified as 2-Ethyl-4-Methyl-1,3-Dioxolan

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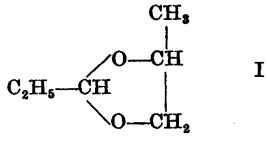
In the course of studies related to addition compounds of ethers we wanted to prepare 2,6- or 2,5-dimethyl-1,4-dioxan. According to Levene and Walti [1] the 2,6-compound is obtained when 1,2-propanediol is distilled with concentrated sulphuric acid. I.G. Farbenindustrie in a patent also states that dimethyl-1,4-dioxan is formed in this way without, however, indicating the positions of the methyl groups [2]. According to another patent, however, of The Standard Oil Development a structural isomeric compound, the cyclic acetal 2-ethyl-4-methyl-1,3-dioxolan (I) is formed under the conditions mentioned above [3].



I

When 1,4-dioxan is prepared by distilling ethylene glycol with sulphuric acid small quantities of a cyclic acetal, *viz.* 2-methyl-1,3-dioxolan, is known to be formed. It therefore appears possible that in the analogous reaction with 1,2-propanediol both dimethyldioxans and 2-ethyl-4-methyl-1,3-dioxolan are formed. The latter may be expected to result from the reaction between 1,2-propanediol and propionaldehyde in the presence of a water-attracting substance like sulphuric acid. Moreover 1,2-propanediol may partially be transformed into propionaldehyde under the conditions prevailing during the distillation process [4]. When fractionating the distillation product considerable quantities of an aldehyde were actually observed.

Levene and Walti believed the substance which they obtained when distilling bis(2-hydroxypropyl)ether with concentrated sulphuric acid to be identical with the substance they prepared from 1,2-propanediol. This was their reason for concluding that the latter is 2,6-dimethyl-1,4-dioxan. The identity of the two liquid products was established only by means of elementary analysis. However, since both dimethyldioxan and the acetal have the formula $C_6H_{12}O_2$ this result is not decisive. On distilling bis(2-hydroxypropyl) ether with sulphuric acid aldehyde was formed. It therefore does not appear impossible that the acetal is produced also under these conditions. The purity of the two products was also somewhat questionable, as the boiling interval reported for the substance obtained from 1,2-propanediol was 117—125°, for the substance obtained from the ether 110—119°.

We also have observed that the product obtained from 1,2-propanediol splits off aldehyde by hydrolysis even after repeated treatment with Tollens' reagent. We further observed that on addition of an acid solution of 2,4-dinitrophenylhydrazine to the substance a precipitate was formed, which was identified as the 2,4-dinitrophenylhydrazone of propionaldehyde. The maximum quantity of the hydrazone obtained by adding a sufficient quantity of 2,4-dinitrophenylhydrazine corresponded to 94 % of the theoretical value calculated under the assumption that the aldehyde is formed by complete hydrolysis of the cyclic acetal. From the products obtained by hydrolysis experiments with diluted acids both 1,2-propanediol and propionaldehyde could indeed be isolated. We have, on the other side, heated a mixture of 1,2-propanediol and propionaldehyde to 160° C in a sealed tube. The substance thus obtained turned out to be identical with the substance resulting from distillation of 1,2-propanediol with sulphuric acid. Their identity follows from the identity of their infrared absorption spectra. We may add that under similar conditions 2,4-dimethyl-1,3-dioxolan has been prepared from 1,2-propanediol and acetaldehyde [5].

According to I. R. investigations the purity of the acetal obtained by the method of I. G. Farbenindustrie for preparing dimethyldioxan is higher than the purity of the products obtained by the methods described by Levene and Walti and in the Standard Oil patent. The main difference is that in the I.G. Farbenindustrie process the 1,2-propanediol is added successively during the distillation. In this case the raw product will — after neutralisation and drying — distill below 117° C, the main part between 114—117° C. The distillation intervals for the purified products reported by Levene and Walti and in The Standard

Oil patent are 117—125° C and 116—121°, respectively.

From our experiments the conclusion must be drawn that *if* dimethyldioxans are formed when propylene glycol is distilled with sulphuric acid, the amounts are at least insignificant.

Experimental: Hydrolysis of the acetal. The acetal is sparingly soluble in water, but on heating with 1 % hydrochloric acid and shaking the acetal layer soon disappeared, propionaldehyde being evolved. The solution was kept for some time in an evacuated desiccator with phosphorus pentoxide and the remaining liquid distilled. The temperature rose rather rapidly to the boiling point of 1,2-propanediol. The distillate was viscous, miscible with water in all proportions and the taste sweet.

Reaction with 2,4-dinitrophenylhydrazine. 5 ml of concentrated sulphuric acid was added to 1.0 g of the reagent, then — drop by drop — 7.5 ml water and finally 25 ml ethanol (96 %). This solution was added to a solution of 0.250 g acetal in 10 ml ethanol (96 %). The precipitate (0.478 g) after repeated crystallisation from 96 % ethanol melted at 153.5°. The melting point showed no depression on addition of specially prepared propionaldehyde-2,4-dinitrophenylhydrazone. (Found: C 45.54; H 4.31; N 23.51. Calc. for $C_9H_{10}O_4N_4$: C 45.37; H 4.23; N 23.53).

The reaction between propionaldehyde and 1,2-propanediol. 17 g of propionaldehyde and 13 g of 1,2-propanediol were heated in a sealed tube to 160° during 68 hours. On fractionating it was found that the liquid distilling below 50° C consisted essentially of propionaldehyde. Between 50° and 86° a two-layer fraction was obtained. Subsequently the temperature rose rapidly to 160° C. The upper layer of the middle fraction was dried with anhydrous sodium sulphate before refractionating. After repeated fractionation the infrared absorption spectrum of the fraction 114—117° was compared with the spectrum of a corresponding fraction obtained from the distillation of 1,2-propanediol with sulphuric acid. The two fractions were found to consist of the same chief component, the contents of free propionaldehyde being somewhat different.

The molecular weight of the acetal prepared according to the I. G. Farbenindustrie patent was determined cryoscopically: Found 115.8, calc. 116.3.

Thanks are due to Mr. J. Lothe for taking the infrared spectra.

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2. I. G. Farbenindustrie D.R.P. 516, 844; *Chem. Zentr.* **1931:1** 2939.
3. Standard Oil Development Co. U.S. 2509, 874; *Chem. Abstracts* **45** (1951) 3867e.
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5. de Gramont, A. *Bull. soc. chim. Paris* [N.S.] **41** (1884) 361.

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A Preliminary Note on the Composition of the Non-hydratable Soyabean Phosphatides

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Degummed soyabean-oil, *i. e.* soyabean-oil from which the "mucilages" and the phosphatides have been removed by treatment with steam or hot water, still contains some phosphorus-containing compounds ordinarily referred to as non-hydratable phosphatides. The composition of these has never been fully established.

From a soyabean-oil extracted from american-grown beans and degummed by treatment with steam the non-hydratable phosphatides corresponding to more than 80 % of the remaining phosphorus-content have been isolated.

The nitrogen-content of the isolated phosphatides was only 0.3 %, whereas the ash-content normally was 21—22 %. The ash consisted mainly of Ca and Mg-phosphates, indicating that the isolated phosphatides were present as Ca and Mg-salts.

After converting the salts into free acids, the ether-soluble part, which contained about 75 % of the phosphorus, was fractionated using the countercurrent technique developed by Craig [1].

Fig. 1 shows the distribution-curves of weight, phosphorus, nitrogen and fatty acids obtained when using the solvent system: 62 volumes of carbon tetrachloride, 35 volumes of methanol and 3 volumes of water [2].

Oil patent are 117—125° C and 116—121°, respectively.

From our experiments the conclusion must be drawn that *if* dimethyldioxans are formed when propylene glycol is distilled with sulphuric acid, the amounts are at least insignificant.

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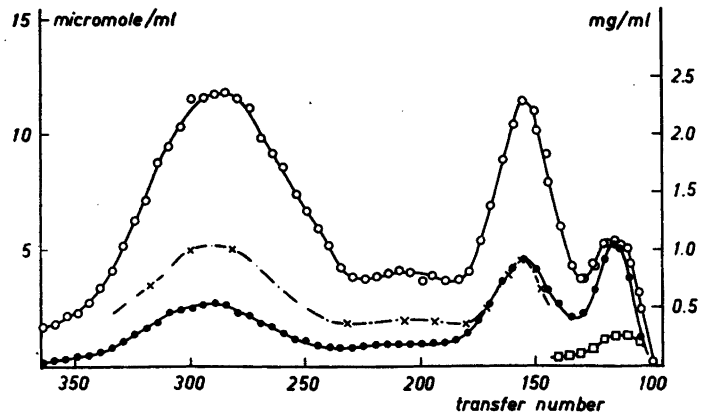

Fig. 1. Distribution curves.

○——○ *weight*, ●——● *phosphorus*,
□——□ *nitrogen*, ×——× *fatty acids*.

The curves exhibit three distinct maxima at transfer Nos. 116, 154 and 285, obviously representing three different components or groups of components, and a less pronounced maximum at transfer No. 205, probably representing a fourth component.

On mild acid hydrolysis the components at transfer No. 116 yielded glycerophosphoric acid and inositolmonophosphoric acid. The latter contained about 12 % of the total phosphorus present at this maximum.

The components at the three other maxima yielded on mild acid hydrolysis glycerophosphoric acid as the only phosphorus-containing ester.

As 1) phosphorus and fatty acids are present in equimolar quantities, 2) nitrogen is not present and 3) glycerophosphoric acid is the only phosphorus-containing ester, the component represented by the maximum at transfer No. 154 is in all probability lysophosphatidic acid (monoacyl-glycerophosphoric acid).

The two maxima at transfer No. 205 and No. 285 both contain phosphorus and fatty acids in a mole ratio of 1:2. As nitrogen is not present and the only phosphorus-containing ester is glycerophosphoric acid, the maxima are thought to represent two groups of phosphatidic acids (diacylglycerophosphoric acids), which are differing in chain length and degree of unsaturation of the fatty acids.

The small nitrogen-content derives from ethanolamine and serine, the latter contains about 10 % of the total nitrogen present. They are in all probability present as esters of phosphoric acid and glycerophosphoric acid.

Based on this distribution, it is possible to state the composition of the investigated mixture, leaving out the nitrogen-containing components, as follows:

inositolmonophosphoric acid	2 %
glycerophosphoric acid	15 %
lysophosphatidic acid	28 %
phosphatidic acid	55 %

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γ-Hydroxyglutamic Acid in Green Plants

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When it was found that the amino acid preparation which Dakin [1] more than 30 years ago isolated from proteins, and which he believed to be β-hydroxyglutamic acid, was a mixture of commonly occurring amino acids [2], hydroxyglutamic acid definitely disappeared from the list of amino acids occurring in nature. We have now, however, found γ-hydroxyglutamic acid in the green parts of *Phlox decussata*.

A 70 % alcohol extract (13 l) of *Phlox* (3.5 kg of fresh plants = 1.18 kg dry substance, = 24.8 g N, = 6.57 g soluble N) was prepared. A two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3) of the extract gave a spot immediately above that of aspartic acid (Fig. 1). In paper electrophoresis the amino acid in question (X) showed to be acidic. The isolation of X was performed as follows: the alcohol extract was passed through an Amberlite IR 120 column. The amino acids remained in the column and were eluted with 1 *N* ammonia. The ammonia was evaporated *in vacuo* and acidic amino acids were separated from the neutral and basic ones in an Amberlite IR-4B column. The acidic amino acids which remained in the column were eluted with 0.4 *N* hydrochloric acid. The solution was evaporated *in vacuo* to a syrup, which was dissolved in 200 ml of water. As the solution still contained great amounts of dark colcuring matter it was neutralized with NaOH. At a pH about 3.5 a dark precipitate was formed. When this precipitate had been separated by filtration the clear but still brown solution was neutralized to pH 7 and once more passed through an Amberlite IR 120 column. This procedure was repeated, ammonia being evaporated in the

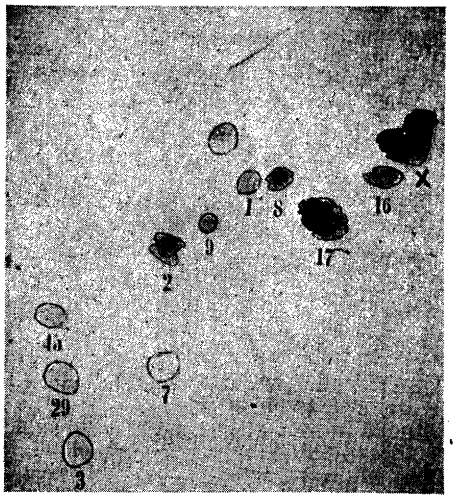

Fig. 1. Two-dimensional paper chromatogram of a 70 % alcohol extract of Phlox decussata. 1 = gly, 2 = ala, 7 = tyr, 8 = ser, 9 = threo, 16 = asp, 17 = glu, X = unknown acidic amino acid.

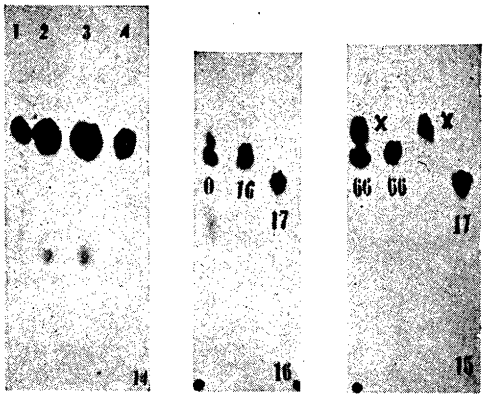

Fig. 2. Paper chromatogram of the pure preparation of X after reduction with HI and red P. 1, 2, and 3 = the reduction products of the amino acid X (increasing concentration of X from 1 to 3), 4 = glutamic acid. Solvent butanol-acetic acid.

Fig. 3. Paper chromatogram of the pure preparation of X after oxidation with $KMnO_4$ in sulphuric acid solution. O = the oxidation products of the amino acid X, 16 = aspartic acid, 17 = glutamic acid. Solvent butanol-acetic acid.

Fig. 4. Paper chromatogram of X and β-hydroxyglutamic acid (synth.). From left: a mixture of X and β-hydroxyglutamic acid (66), pure β-hydroxyglutamic acid (66), pure X and pure glutamic acid.

meantime. The solution was finally evaporated to dryness *in vacuo*, and the light solid substance remaining in the flask was dissolved in a small amount of water. 5 g of cellulose powder was poured into the flask after which the mass was evaporated to dryness, and placed on the top of a cellulose powder column (5.5 × 110 cm) containing as indicator 20 mg of methylorange. The elution was performed with a solution of butanol-acetic acid. After the indicator had come out 125 fractions of 36 ml were taken. Fractions 45—62 contained glutamic acid, 55—99 aspartic acid, and 71—125 the amino acid X. Fractions 100—125 contained only the unknown amino acid. They were evaporated to dryness *in vacuo*, the substance was dissolved in water and the solution poured into a glass jar. The solution was evaporated on a waterbath until crystallization began. After cooling the crystalline substance was separated by centrifugation and dried in a vacuum desiccator. The yield was 124 mg. M.p. 187° C (decomp.). Fractions 71—99 contained, besides a small amount of aspartic acid, about 620 mg of the amino acid X. 3.5 kg of fresh *Phlox* (= 6.57 g sol. N) thus contained about 750 mg (65 mg N) of the unknown amino acid (1.0 % of soluble N). Through crystallization a part of the amino acid X in fractions 71—99 could be separated from aspartic acid.

By the following experiments we established the structure of the amino acid X:

1. By reduction with 66 % HI (*d* 1.96) and red phosphorus at 140° C for 4 h glutamic acid was found to be formed (Fig. 2) from which we conclude that the amino acid X is a hydroxyglutamic acid.

2. Analysis of the amino acid X: C 36.82; H 5.35; N 8.60. Calc. for $C_5H_9O_5N$: C 36.81; H 5.52; N 8.59. Titration with phenolphtalein as indicator: 2.281 mg used 1.36 ml 0.00977 *N* NaOH. Calc. for monohydroxyglutamic acid 1.43 ml. The amino acid thus has the same empirical formula as monohydroxyglutamic acid.

3. Upon heating the amino acid X at pH 3.3 and 125° C for 4 h the amino N (van Slyke) diminished by 95 %; accordingly hydroxypyrrolidonecarboxylic acid was formed. From glutamic acid pyrrolidonecarboxylic acid is formed correspondingly (decrease of amino N 90 %).

4. By oxidation with potassium permanganate in sulphuric acid solution (10 mg of X + 7.5 mg of $KMnO_4$ in 189 µl of water + 90 µl of 20 % H_2SO_4) at + 10° C aspartic acid was formed (Fig. 3).

5. Potentiometric titration in parallel experiments gave the following pK-values (values approximate, taken from titration curves).

	pK_2	pK_3
Glutamic acid	4.2	9.7
Aspartic acid	3.7	9.6
β-Hydroxyglutamic acid	4.2	9.3
γ-Hydroxyglutamic acid (X)	3.6	9.7

6. Synthetic β-hydroxyglutamic acid can be distinguished from our γ-hydroxyglutamic acid on the paper chromatogram (butanol + acetic acid) (Fig. 4).

	butanol + acetic-acid	phenol + NH_3
R_F-value for γ-hydroxyglutamic acid (X)	0.06	0.17
» » β-hydroxyglutamic acid (synth.)	0.09	0.17
» » γ-hydroxy-α-amino pimelic acid [3]	0.15	0.34
» » glutamic acid	0.14	0.31

The new amino acid found in *Phlox* has thus been established as γ-hydroxyglutamic acid, HOOC · CHOH · CH_2 · $CHNH_2$ · COOH. On the basis of the paper chromatogram *Linaria vulgaris* also contains γ-hydroxyglutamic acid. The acid has not been isolated from this plant.

γ-Hydroxyglutamic acig is found also in the protein fraction of *Phlox*.

We are very grateful to Dr. Karl Pfister, Rahway, New Jersey, for a preparation of synthetic β-hydroxy-glutamic acid.

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Oxidation of Diphenylmethane and Benzyltoluene with Dilute Nitric Acid at High Temperature and Pressure

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Senff [1] boiled *m*-benzyltoluene for 4 days with dilute nitric acid and obtained an impure *m*-benzylbenzoic acid. In a similar way Zincke [2] prepared *p*-benzylbenzoic acid from *p*-benzyltoluene and obtained *p*-toluenephenylketone as a by-product. Rivkin [3] boiled diphenylmethane for several hours with 65 % HNO_3 and obtained benzophenone in good yield together with α-nitrodiphenylmethane. Nothing is mentioned about ring nitrated by-products.

It has now been shown that the methylene group in diphenylmethane and benzyltoluene can be oxidized to a CO-group in high yields and in a very short reaction time if dilute nitric acid is used at high temperature and pressure [4]. The oxidation is performed with nitric acid of a concentration around 30 % at temperatures around 200° C and pressures around 30 kg/cm².

A very quick reaction occurs under such conditions and benzyltoluene can be converted to crude benzoylbenzoic acid in yields of 90 % or more. The oxidation can be carried out in a very convenient way from a technical point of view. By treating the mixture of benzoylbenzoic acid isomers with hot conc. sulphuric acid the *o*-isomer is converted to anthraquinone [5]. (The *p*-isomer can be esterified to give valuable plasticizers.) It seems not impossible that this route to anthraquinone might have economic possibilities, provided low-priced benzyltoluene is available. Benzyltoluene is a by-product from the manufacture of benzaldehyde by oxidation of toluene with MnO_2 and can also be obtained as the main product from this oxidation [6].

Experimental: Oxidation of diphenylmethane. The oxidations were performed in an autoclave of stainless steel according to a general procedure reported in a previous paper [7]. Diphenylmethane and nitric acid of 30 % concentration in a 30 % equivalent excess were heated in the autoclave with stirring to 150° C. At this temperature the reaction started and no more external heating was needed. The pressure rose to 25 kg/cm² and the temperature to 195° C in a few minutes. Then the temperature was kept around 195° C for approximately 15 minutes. After cooling the reaction mixture was filtered (the mother liquid contained 6.1 % HNO_3), and the reaction product was washed with water and dilute Na_2CO_3-solution. It was then fractionated to give a main fraction with the analytical data tabulated in Table 1. As a comparison, data on benzophenone and diphenylmethane from the literature have been tabulated.

Table 1.

	Oxidation product	Benzo-phenone	Diphenyl-methane
Boiling point °C	B.p.$_5$ 150	B.p.$_{10}$161	B.p.$_{11}$120
Density D_4^{20}	1.1097	1.1108	1.0060
Refract. index	n_D^{20} 1.6080	n_D^{19}1.6077	n_D^{20}1.5768
C %	85.1	85.8	92.9
H %	4.9	5.5	7.1

Oxidation of benzyltoluene. Benzyltoluene (mixture of isomers, $D_4^{20}=0.999$, $n_D^{20}=1.5723$) 72 g, and HNO_3 of 15 % concentration in a 20 % equivalent excess (for the oxidation of benzyltoluene to benzoylbenzoic acid and reduction of HNO_3 to NO), were heated in a 2.5 liter autoclave. When the reaction had started, the temperature rose to 200° C and the pressure to 25 kg/cm² in a few minutes. The autoclave was then allowed to cool off and after filtering and washing with water and drying a yellow product was obtained. This was dissolved in NaOH of 10 % concentration and after filtration reprecipitated with HCl. The yield was 72 g. Part of the crude product (20 g) did not dissolve in NaOH (melting point 104—109° C). Another experiment (No. 2) was run in the same way but using a 50 % excess of nitric acid. The temperature rose to 203° C and the pressure to 32 kg/cm². This product was not reprecipitated from the Na-salt but analysed directly after washing with water and drying. Yield 73 %. The values may be seen from Table 2. As a comparison theoretical values for benzoylbenzoic acid are tabulated.

Another three experiments were run in the same way at the same temperature and pressure as No. 2. The mother liquid from these experiments contained 3.4, 3.5 and 4.2 % HNO_3, respectively, and the crude acids contained 0.7—0.9 % N (Dumas) and had the equivalent weights 231.4, 232.5 and 246.6,

Table 2.

	Crude product No. 1	No. 2	Theoretical data
Equiw. weight	231	228	226
N %	0.9	0.8	—
C %	72.9	72.0	74.3
H %	4.3	4.3	4.4
Ash %	0.12	0.29	—

respectively. The average yield of crude product was 88 %.

Also tried was the oxidation of benzyltoluene according to this method, without excess of nitric acid. Using 91 g benzyltoluene and the equivalent amount of HNO_3 a temperature of 206° C and a pressure of 22 kg/cm² were reached. The reaction product was oily and could be separated into 26 g of solid material and 70 g of oil. After washing with benzene the solid material weighed 11 g and had the equivalent weight 237 and melting point 192—193° C. (Literature value for *p*-benzoylbenzoic acid 194° C.)

Finally some experiments were run with HNO_3 of 15 % concentration in a 50 % excess but this time the nitric acid was charged in two portions. Half of the acid was charged from the beginning and at 135—140° C the temperature and pressure began to rise quickly, the temperature reaching 180° C and the pressure 30—40 kg/cm² in a few minutes. When the temperature and pressure failed to rise any more but started to decrease, the other half of the nitric acid was placed into the autoclave and the temperature was brought up to about 200° C by external heating. The reaction was comparatively slow during this second phase. Operating this way an increase in capacity was possible. In each experiment 182 grams of benzyltoluene was oxidized in a 2.5 liter autoclave. The result of this series may be seen from Table 3. As usual the figures for temperature and pressure refer to maximal values.

Table 3.

Temperature °C	Pressure Kg/cm²	Yield %	Equiv. weight	N (Dumas) %
197	34	94.0	225.7	0.6
201	36	93.0	228.2	0.8
200	39	97.5	234.6	0.7
198	40	88.5	218.4	0.7

Such a crude acid mixture was purified with SO_2 in alkaline solution according to a procedure described in a previous paper [8] and was then free from nitro compounds. (Yield 79 %). It was heated (100 g) in 96 % H_2SO_4 at 130° C for 1 hour and after cooling the solution was diluted with water. After washing with water the precipitate was treated with Na_2CO_3. The part of the product which was not soluble in Na_2CO_3 was washed with water and dried. The melting point was 278° C. (Literature values for antraquinone 273—286° C.) Yield 41 g. The product was identified in ultraviolet spectrophotometer as antraquinone. The Na_2CO_3-solution was acidified with H_2SO_4 and 47 g of a product, melting at 190° C was obtained. (Literature value for *p*-benzoylbenzoic acid 194° C.)

The author wishes to thank Dr. Allan Dahlén and Dr. Bo af Ekenstam for their helpful interest in this work and for valuable discussions.

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1,2-Dithiolane-3-carboxylic Acid

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As early as 1938 Fredga [1,2] had studied α,α′-dimercaptoadipic acid and its cyclic oxidation product 1,2-dithiane-3,6-dicarboxylic acid and pointed out the importance of studying aliphatic α,α′-dimercapto dicarboxylic acids and their oxidation to cyclic disulphides.

Schotte has continued the investigation and prepared 1,2-dithiolane-3,5-dicarboxylic acid (1,2-dithia*cyclo*pentane-3,5-di-

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Ash %	0.12	0.29	—

respectively. The average yield of crude product was 88 %.

Also tried was the oxidation of benzyltoluene according to this method, without excess of nitric acid. Using 91 g benzyltoluene and the equivalent amount of HNO_3 a temperature of 206° C and a pressure of 22 kg/cm² were reached. The reaction product was oily and could be separated into 26 g of solid material and 70 g of oil. After washing with benzene the solid material weighed 11 g and had the equivalent weight 237 and melting point 192—193° C. (Literature value for *p*-benzoylbenzoic acid 194° C.)

Finally some experiments were run with HNO_3 of 15 % concentration in a 50 % excess but this time the nitric acid was charged in two portions. Half of the acid was charged from the beginning and at 135—140° C the temperature and pressure began to rise quickly, the temperature reaching 180° C and the pressure 30—40 kg/cm² in a few minutes. When the temperature and pressure failed to rise any more but started to decrease, the other half of the nitric acid was placed into the autoclave and the temperature was brought up to about 200° C by external heating. The reaction was comparatively slow during this second phase. Operating this way an increase in capacity was possible. In each experiment 182 grams of benzyltoluene was oxidized in a 2.5 liter autoclave. The result of this series may be seen from Table 3. As usual the figures for temperature and pressure refer to maximal values.

Table 3.

Temperature °C	Pressure Kg/cm²	Yield %	Equiv. weight	N (Dumas) %
197	34	94.0	225.7	0.6
201	36	93.0	228.2	0.8
200	39	97.5	234.6	0.7
198	40	88.5	218.4	0.7

Such a crude acid mixture was purified with SO_2 in alkaline solution according to a procedure described in a previous paper [8] and was then free from nitro compounds. (Yield 79 %). It was heated (100 g) in 96 % H_2SO_4 at 130° C for 1 hour and after cooling the solution was diluted with water. After washing with water the precipitate was treated with Na_2CO_3. The part of the product which was not soluble in Na_2CO_3 was washed with water and dried. The melting point was 278° C. (Literature values for antraquinone 273—286° C.) Yield 41 g. The product was identified in ultraviolet spectrophotometer as antraquinone. The Na_2CO_3-solution was acidified with H_2SO_4 and 47 g of a product, melting at 190° C was obtained. (Literature value for *p*-benzoylbenzoic acid 194° C.)

The author wishes to thank Dr. Allan Dahlén and Dr. Bo af Ekenstam for their helpful interest in this work and for valuable discussions.

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1,2-Dithiolane-3-carboxylic Acid

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As early as 1938 Fredga [1,2] had studied α,α′-dimercaptoadipic acid and its cyclic oxidation product 1,2-dithiane-3,6-dicarboxylic acid and pointed out the importance of studying aliphatic α,α′-dimercapto dicarboxylic acids and their oxidation to cyclic disulphides.

Schotte has continued the investigation and prepared 1,2-dithiolane-3,5-dicarboxylic acid (1,2-dithia*cyclo*pentane-3,5-di-

carboxylic acid) [3] and 1,2-dithia*cyclo*heptane-3,7-dicarboxylic acid [4].

The expectations of Fredga [1] have been realised by the discovery of "6-thioctic acid" (6,8-dithio-*n*-octanoic acid) and the important role this acid plays as a coenzyme [5] and in the photosyntheses [6]. It is the author's intention to synthesize lower homologues of 6-thioctic acid, to study the labile 1,2-dithiolane ring and to investigate the characteristics of these compounds as a function of the length of the side chain. The investigation is in progress. This publication deals with the synthesis of *rac.*-1,2-dithiolane-3-carboxylic acid and preliminary experiments on its optical resolution.

Methyl α,γ-dibromo-butyrate (prepared from α,γ-dibromo-butyryl bromide [7]) was treated with potassium thiol-acetate [8] and hydrolyzed with alkali to give α,γ-dimercapto-butyric acid. Oxidation of the mercapto-acid was carried out with iodine [9], oxygen [10] and hydrogen peroxide, but mostly polymeric products were formed. Experiments on oxidation to get a higher yield of the monomeric five-membered cyclic disulphide are in progress. The dithiolane-carboxylic acid was extracted with benzene in which the polymeric products are insoluble. Extraction with ether is unsuitable because part of the polymers is soluble in that solvent. The acid was obtained as a beautifully crystalline product of pale-yellow colour. On heating it is readily polymerized to a tough rubber-like mass. Attempts to distil the acid in vacuum also gave a polymeric mass.

Reduction of 1,2-dithiolane-3-carboxylic acid with zinc powder in ammoniacal solution yielded dimercapto-butyric acid. This indicates that the structure $>S=S$ for the disulphide group is excluded.

1,2-dithiolane-3-carboxylic acid was obtained in two different crystalline forms having the melting points 80–82° and 147°. Their ultraviolet absorption spectra are identical, with an absorption peak at 280 mμ, which means a shift from λ_{max} 330 mμ for 1,2-dithiolane and 6-thioctic acid. When another carboxylic group is inserted, the shift to shorter wavelengths is still greater. Schotte [11] has found that 1,2-dithiolane-3,5-dicarboxylic acid has λ_{max} 250 mμ.

The dissociation constant of the acid was preliminarily determined by measuring the pH of a solution of equivalent quantities of the acid and its sodium salt.

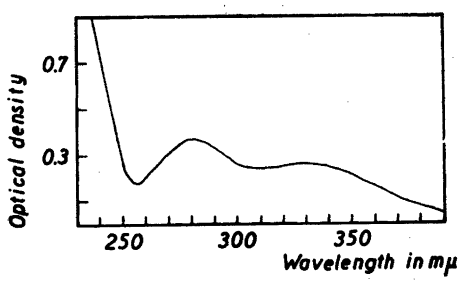

Fig. 1. Ultraviolet absorption spectra of 1,2-dithiolane-3-carboxylic acid in 95 % ethanol, 1.55 × 10⁻³ M.

Preliminary experiments on resolution have been carried out. With brucine and quinidine no crystals could be obtained and (+)-α-phenylethylamine gave a salt containing inactive acid. A dextrorotary acid was, however, obtained from the cinchonidine salt, crystallized from dilute alcohol. The mother liquor yielded crystals of different form containing excess of (−)-acid.

Experimental. Methyl α,γ-dibromobutyrate. The ester was obtained from abs. methanol and α,γ-dibromobutyryl bromide (B.p. 106—107°/9 mm. Plienninger gives no boiling point) in quantitative yield. B.p. 101—102°/9 mm.

Methyl-α,γ-di-thiolacetoxy-butyrate. 139 g of methyl α,γ-dibromobutyrate in 200 ml methanol was treated with an ethanolic solution of potassium thiolacetate prepared by neutralizing 90 g of thiolacetic acid with a solution of 66 g of potassium hydroxide in 200 ml methanol. There was an immediate precipitation of potassium bromide, which after standing overnight was filtered off and the methanol evaporated. Water was added, and the oil extracted with ether. The dried ethereal solution was evaporated and distilled to give 107 g (80 %) of pale yellow ester b.p. 178—179°/10 mm. About 10 gram distilled over below 178° and was shown to contain bromine.

α,γ-Dimercapto-butyric acid. 55 g of the ester was treated with 800 ml (675 ml of methanol and 125 ml of water) 2 *N* sodium hydroxide. After standing over-night, the methanol was distilled at 760 mm in nitrogen atmosphere in order to avoid oxidation. By treating the residue with dilute sulfuric acid and extracting with ether 38 g crude mercapto-acid was obtained as a yellow oil. The acid contained some acetic acid.

1,2-Dithiolane-3-carboxylic acid. The crude mercapto-acid was neutralized with 1 000 ml of dilute sodium-bicarbonate solution, and 2 ml 1 % ferric chloride was added, resulting in a solution of deep blue colour. With mechanical stirring and cooling with ice, 30 % hydrogen peroxide was added until the blue colour disappeared. The solution was acidified with sulfuric acid and extracted with benzene. (Part of the product was insoluble in benzene, but could be extracted with ether.) The benzene solution was dried and distilled. The crystallized residue weighed 5 g (15 % calculated on methyl α,γ-di-thiolacetoxy-butyrate). Recrystallization from *cyclo*hexane-benzol gave very pale yellow crystals m. p. 81—82°. (Found: Eq. wt. 150.6; S 42.61; Mol. wt. 147. Calc. for $C_4H_8O_2S_2$: Eq. wt. 150.2; S 42.69; Mol. wt. 150.2.) The molecular weight was determined ebullioscopically in acetone. From the acetone solution the acid was recovered as large crystals m. p. 147°.

Oxidation with gaseous oxygen using ferric chloride as catalyst and oxidation with iodine gave about the same yield as the peroxide oxidation.

Reduction. The polymeric acid was almost quantitatively reduced to the mercapto-acid with zinc powder [1]. (Titr. with NaOH and I_2 gave: Eq. wt. 152.8 and 160.1; Calc. for $C_4H_8O_2S_2$: Eq. wt. 152.2.)

Preliminary experiments on resolution. 0.15 g (0.001 mole) of rac. 1,2-dithiolane-3-carboxylic acid and 0.29 g (0.001 mole) of cinchonidine were dissolved in 2 ml ethanol and 1 ml water was added. Crystallization overnight yielded 0.20 g of salt. The acid was liberated from its salt in the usual manner. The rotatory power was measured in alcohol. M. p. 60—62°.

$$[\alpha]_D^{25} = + 158°$$

Preliminary value of the dissociation constant. In a solution of 1.090×10^{-4} moles of 1,2-dithiolane-3-carboxylic acid and 1.090×10^{-4} moles of its sodium salt in 15 ml water the pH was found to be 3.42.

The author wishes to express his gratitude to Professor Arne Fredga, who suggested this problem, for valuable advice and stimulating discussions.

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Crystal Field Stabilization of First Transition Group Complexes

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The crystal field of Bethe [1] has been applied to the first transition group by Ilse and Hartmann [2], Santen and Wieringen [3] and Orgel [4]. Ballhausen and the present authors [5-8] have extended these applications, and in this communication special attention will be drawn to the energy decrease due to crystal field perturbations on complex ions with an unfilled 3d-shell.

Table 1 gives the data for crystal fields with cubic symmetry (originating from octahedral and tetrahedral complexes). In the cases where the complexes with different total spin quantum number are known, also the lowest value of S observed in complexes is considered. The stabilization parameter is defined: $\varrho = 2a - 3b$, where a is the number of γ_5- and b the number of γ_3-electrons in a strong crystal field of cubic symmetry [3,8]. The two states γ_5 and γ_3 of one d-electron have the energies E_2 and E_1 respectively [2]. The energy difference $(E_1 - E_2)$ in tetrahedral complexes [5] equals $-\frac{4}{9}(E_1 - E_2)$ in octahedral complexes. Thus, the tetrahedral configuration gives much lower crystal field influences than the corresponding octahedral configuration. The energy of the groundstate in terms of ϱ is decreased by $\frac{\varrho}{5}(E_1 - E_2)$ relative to a similar ion of spherical symmetry, *i. e.* a closed shell with $L = 0$. Since $(E_1 - E_2)$ in octahedral complexes in the first transition group is of the order of magnitude [6] 10 000 cm^{-1}

1,2-Dithiolane-3-carboxylic acid. The crude mercapto-acid was neutralized with 1 000 ml of dilute sodium-bicarbonate solution, and 2 ml 1 % ferric chloride was added, resulting in a solution of deep blue colour. With mechanical stirring and cooling with ice, 30 % hydrogen peroxide was added until the blue colour disappeared. The solution was acidified with sulfuric acid and extracted with benzene. (Part of the product was insoluble in benzene, but could be extracted with ether.) The benzene solution was dried and distilled. The crystallized residue weighed 5 g (15 % calculated on methyl α,γ-di-thiolacetoxy-butyrate). Recrystallization from *cyclo*hexane-benzol gave very pale yellow crystals m. p. 81—82°. (Found: Eq. wt. 150.6; S 42.61; Mol. wt. 147. Calc. for $C_4H_8O_2S_2$: Eq. wt. 150.2; S 42.69; Mol. wt. 150.2.) The molecular weight was determined ebullioscopically in acetone. From the acetone solution the acid was recovered as large crystals m. p. 147°.

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Table 1. Stabilization of ground-states in strong crystal fields of cubic symmetry.

Electron number	Examples	Total spin S	Octahedral complexes Stabilization parameter ϱ	Octahedral complexes Orbital degeneracy e	Tetrahedral complexes Stabilization parameter $-\varrho$	Tetrahedral complexes Orbital degeneracy e
d^0	Ca++, Sc+++	0	0	1	0	1
d^1	Ti+++	$\frac{1}{2}$	2	3	3	2
d^2	V+++	1	4	3	6	1
d^3	V++, Cr+++	$\frac{3}{2}$	6	1	4	3
d^4	Cr++, Mn+++	2	3	2	2	3
		1	8	3	7	3
d^5	Mn++, Fe+++	$\frac{5}{2}$	0	1	0	1
		$\frac{1}{2}$	10	3	10	3
d^6	Fe++, Co+++	2	2	3	3	2
		0	12	1	8	
d^7	Co++	$\frac{3}{2}$	4	3	6	1
		$\frac{1}{2}$	9	2	6	
d^8	Ni++	1	6	1	4	3
		0	6	2	4	3
d^9	Cu++	$\frac{1}{2}$	3	2	2	3
d^{10}	Zn++, Ga+++	0	0	1	0	1

(= 28 kcal/mole) with divalent ions and 20 000 cm^{-1} with trivalent ions, this stabilization energy can be considerable [4] even though it must be subtracted from the energy of the (often intermixed) excited states of the free ion in the magnetically anomalous complexes.

Another feature of the stabilization first mentioned by Orgel [4] will be discussed in this note: The preference for γ_5-electrons in the octahedral complexes corresponds to high electron densities in the eight directions [1] between the ligands, while the missing γ_3-electrons are directed towards the six ligands. Thus, the electrostatic potential near the ligands will be higher than corresponding to the external charge of the complex, *e. g.* in diamagnetic cobalt(III) complexes, the effective external charge might be expected to vary between + 9 in the direction of the ligands and −3 between the ligands. But a closer electrostatic calculation shows that the screening is not only determined by the number of d-electrons situated directly between the ligand and the cobalt(III) nucleus. Actually, only a variation between +4 and +2 can be present. Nevertheless, the result supports the idea that complexes like $[Co(NH_3)_6]^{+++}$ can be held together by electrostatic forces.

The current theories [9] on covalency in coordination compounds assume donation of twelve electrons from the ligands to the central ion in octahedral complexes, forming hybridizations of the d^2sp^3-type. It does not seem accidental that precisely the two empty γ_3-orbitals are used for this operation. The deviation from the spherical symmetry of the d-shell (*i. e.* ϱ being $\neq 0$) corresponds to a preferred location of ligands in the directions of the empty γ_3-orbitals, which are the spherically symmetric complement to the filled γ_5-orbitals. In the authors' opinion, it does not seem necessary to explain the electrostatic energy decrease by further addition of electron-pair formation of donated character. The tetrahedral sp^3-hybridizations, which are said to occur in ions with no crystal field perturbations, constitute as peculiar a hypothesis in coordination chemistry. While the explanation of chemical bonding between four hydrogen atoms each with one un-paired electron and a carbon atom with four un-paired electrons is so successful, the similar treatment of zinc(II) or beryllium(II) complexes does not easily compete with the electrostatic picture according to Fajans [10].

In Table 1, the orbital degeneracy e is also given for all the groundstates except

when it cannot be certainly predicted from atomic spectroscopy. Only in the cases for which $e = 1$ are the complexes stable [11] for crystal fields of purely cubic symmetry. For other e values the crystal fields must be somewhat distorted; for $e = 2$, to tetragonal, and for $e = 3$, to rhombic or tetragonal symmetry. Crystal field calculations explain easily [5,7] the occurrence of tetragonal symmetry in copper(II) or in diamagnetic nickel(II) complexes with no need of dsp^2-hybridization. If complexes are classified according to $e = 1$ and $e = 2$ or 3, it is interesting to compare the absorption spectra as well as the consecutive complexity constants in the various cases. Thus displacements of bands towards lower wavenumbers by increasing symmetry of the crystal field are found only in systems with $e > 1$ (*e. g.* in the cupric ammonia system) and irregularities in the rest effects are found *e. g.* to a higher degree in the cobalt(II) ammonia system than in the nickel(II) system [12,p.67].

The activation energies are high only when $\varrho(E_1 - E_2)$ is large and $e = 1$. Bjerrum and Poulsen [13] found empirically that half-completed and completed d-shells (with $\varrho = 0$) and nearly complete d-shells (with $e > 1$) give low activation energies, which increase with the oxidation state for purely electrostatic reasons, just as do the complexity constants. In the robust complexes (*e.g.* $\varrho = 6$ and $e = 1$ in chromium (III) and nickel(II)) the activation energy increases with the value of $(E_1 - E_2)$, which [6] is 17 400 cm^{-1} in $[Cr(H_2O)_6]^{+++}$ and 7 600 cm^{-1} in $[Ni(H_2O)_6]^{++}$. If the magnetism is changed during a consecutive complex formation, a sudden change of activation energies and complexity constants are expected, as found in the cyanide and α,α'-dipyridyl complexes of iron(II)[14]. Magnetically anomalous, octahedral complexes should only be possible when $\frac{\varrho}{5}(E_1 - E_2)$ is larger than the energy of the excited states in the free ion with low S, and they are mainly restricted to the electron configurations d^5 and d^6 with the highest value of ϱ. These facts will be discussed in the following papers of the series "Studies of Absorption Spectra"[15].

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Note on the Structure of Dimethoxydihydrofurfural Diacetate

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By the action of bromine in methanol on furfural diacetate Clauson-Kaas and Fakstorp [1] obtained an 81 % yield of an oily dimethoxydihydrofurfural diacetate which by hydrolysis and oxidation with periodic acid gave *cis*-β-formylacrylic acid. Therefore the product must be a derivative of 2-pentene-1,5-dial-4-one (II) and it was assumed to have structure I in analogy with other dimethoxydihydrofurans.

When repeating the synthesis of dimethoxydihydrofurfural diacetate a somewhat lower yield (about 65 %) was regularly obtained while the reaction product at the same time contained about 15 % of unreacted furfural diacetate. Since the methoxylation of furfural diacetate is a slower reaction than the usual furan methoxylation reactions, the discrepancy may be due to a catalytic effect.

when it cannot be certainly predicted from atomic spectroscopy. Only in the cases for which $e = 1$ are the complexes stable [11] for crystal fields of purely cubic symmetry. For other e values the crystal fields must be somewhat distorted; for $e = 2$, to tetragonal, and for $e = 3$, to rhombic or tetragonal symmetry. Crystal field calculations explain easily [5,7] the occurrence of tetragonal symmetry in copper(II) or in diamagnetic nickel(II) complexes with no need of dsp^2-hybridization. If complexes are classified according to $e = 1$ and $e = 2$ or 3, it is interesting to compare the absorption spectra as well as the consecutive complexity constants in the various cases. Thus displacements of bands towards lower wavenumbers by increasing symmetry of the crystal field are found only in systems with $e > 1$ (*e. g.* in the cupric ammonia system) and irregularities in the rest effects are found *e. g.* to a higher degree in the cobalt(II) ammonia system than in the nickel(II) system [12,p.67].

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Note on the Structure of Dimethoxydihydrofurfural Diacetate

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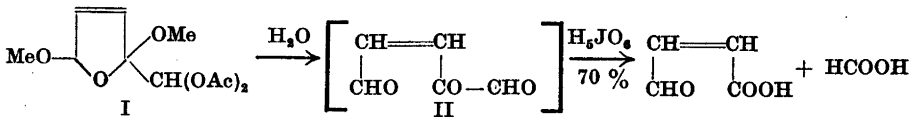
By the action of bromine in methanol on furfural diacetate Clauson-Kaas and Fakstorp [1] obtained an 81 % yield of an oily dimethoxydihydrofurfural diacetate which by hydrolysis and oxidation with periodic acid gave *cis*-β-formylacrylic acid. Therefore the product must be a derivative of 2-pentene-1,5-dial-4-one (II) and it was assumed to have structure I in analogy with other dimethoxydihydrofurans.

When repeating the synthesis of dimethoxydihydrofurfural diacetate a somewhat lower yield (about 65 %) was regularly obtained while the reaction product at the same time contained about 15 % of unreacted furfural diacetate. Since the methoxylation of furfural diacetate is a slower reaction than the usual furan methoxylation reactions, the discrepancy may be due to a catalytic effect.



On standig, a crystalline isomer was precipitated from the oily dimethoxydihydrofurfural diacetate (yield 14 %). Treatment of this crystalline dimethoxydihydrofurfural diacetate with sodium methoxide in methanol gave a liquid with the formula $C_5H_4O_2(OMe)_2$. Catalytic hydrogenation gave a liquid $C_5H_6O(OMe)_2(OAc)_2$, which with sodium methoxide gave a third liquid $C_5H_6O_2(OMe)_2$. We think that these reactions are best explained as shown below, where structure I (either the *cis* or the *trans* form) is assigned to the crystalline dimethoxydihydrofurfural diacetate, while the three liquids are proposed to be the new 2,5-dimethoxy-2,5-dihydrofurfural (III), 2,5-dimethoxytetrahydrofurfural diacetate (IV) and 2,5-dimethoxytetrahydrofurfural (V), all having the same steric configuration as I at the α-carbons.

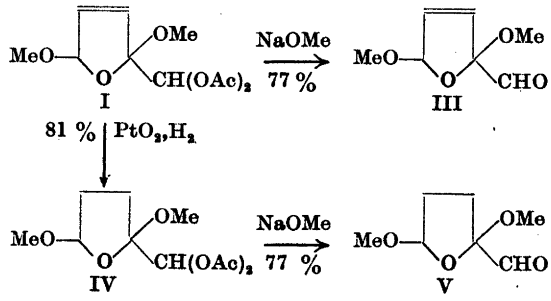
The mother liquor from the crystalline dimethoxydihydrofurfural diacetate (I) gave, with sodium methoxide, a dark red solution, from which 9 % of the liquid $C_5H_4O_2(OMe)_2$ (2,5-dimethoxy-2,5-dihydrofurfural (III)) was isolated. The main part of the reaction product remained as a red mass. Very probably III is formed from a certain amount (about 11 %) of I, which has remained in the mother liquor, the total yield of I thus being about 22 %. The major component of the mother liquor can, on the other hand, not be one of the two stereoisomeric forms of I, but must be another derivative of 2-pentene-1,5-dial-4-one (II). So far we have not been able to elucidate its structure.

Experimental. (Microanalyses by E. Boss and K. Glens). *Isolation of crystalline dimethoxydihydrofurfural diacetate (I).* Furfural diacetate (39.6 g) was methoxylated as described by Clauson-Kaas and Fakstorp [1]. The yield of the reaction product (slightly yellow oil, b.p.$_2$ 119—125°, n_D^{25} 1.4494) was 31.1 g (60 %, three other preparations gave 61, 63 and 67 %) (Found: C 50.8; H 6.4; OCH_3 20.4; $COCH_3$ 34.6. Calc. for $C_5H_4O_2(OCH_3)_2(COCH_3)_2$ (260.2) containing 15 % of $C_5H_4O_2(COCH_3)_2$ (198.2): C 51.3; H 6.0; OCH_3 20.4; $COCH_3$ 34.7).

30.0 g of the reaction product was left standing at room temperature for five weeks, a precipitate of white crystals of I removed by filtration (4.26 g = 14 %) and the filtrate (n_D 1.4498) analysed (Found: C 50.8; H 6.4; OCH_3 19.0; $COCH_3$ 34.8. Calc. for $C_5H_4O_2(OCH_3)_2(COCH_3)_2$ (260.2) containing 18 % of $C_5H_4O_2(COCH_3)_2$ (198.2): C 51.5; H 6.0; OCH_3 19.6; $COCH_3$ 34.9).

The crystals were crystallized from methanol (4.5 ml) and washed twice with methanol (4 ml in all). Hereby 3.04 g of a product melting at 110—111° (Hershberg apparatus corr.) was obtained. Further crystallization did not change the m.p. (Found: C 50.8; H 6.3; OCH_3 23.6; $COCH_3$ 33.5. Calc. for $C_5H_4O_2(OCH_3)_2(COCH_3)_2$ (260.2): C 50.8; H 6.2; OCH_3 23.9; $COCH_3$ 33.1).

The acetyl determinations were carried out by acidimetric titration at room temperature (phenolphthalein indicator). In the usual determinations (distillation of acetic acid from a 48 % sulfuric acid solution) acetyl values about 30 % higher than the calculated amount were obtained. We believe this to be due to hydrolytic cleavage of 2-pentene-1,5-dial-4-one (II) with the formation of formic acid.



2,5-Dimethoxy-2,5-dihydrofurfural (III). I (3.01 g, 0.116 mole) was dissolved in methanol (10 ml) and the solution added to a solution of sodium methoxide (from 0.266 g of sodium; 0.0116 mole) in methanol (15 ml). After standing at room temperature for 15 minutes the methanol and the methyl acetate formed by the reaction were distilled in a vacuum. Ether (50 ml) was added to the colorless residue, a precipitate of sodium acetate (0.88 g = 93 %) removed by filtration and the ether solution distilled. The yield was 1.40 g (77 %) of III (colorless liquid, b.p.$_{10}$ 88°, n_D^{25} 1.4430) (Found: C 53.3; H 6.8; OCH_3 39.6. Calc. for $C_5H_6O_2(OCH_3)_2$ (158.2): C 53.2; H 6.4; OCH_3 39.3).

On standing the product changes into a more viscous liquid with a higher refractive index. Probably polymerization takes place. Redistillation of 1.20 g after 10 days gave 0.74 g of the original product (b.p.$_{20}$ 98°, n_D^{25} 1.4431).

2,5-Dimethoxytetrahydrofurfural diacetate (IV). I (2.34 g) was dissolved in dioxan (50 ml) and the solution shaken (3 hr) with Adams platinum oxide catalyst (100 mg) under hydrogen (70 atm). The product was isolated by distillation. The yield was 1.92 g (81 %) of IV (colorless liquid, b.p.$_{0.3}$ 97—99°, n_D^{25} 1.4398) (Found: C 50.7; H 7.2; OCH_3 23.5; $COCH_3$ 31.8. Calc. for $C_5H_6O_3(OCH_3)_2(COCH_3)_2$ (262.3): C 50.4; H 6.9; OCH_3 23.7; $COCH_3$ 32.8).

2,5-Dimethoxytetrahydrofurfural (V). IV (3.00 g) was treated with sodium methoxide as described above for the preparation of III. The yield was 1.40 g (77 %) of V (slightly yellow liquid, b.p.$_{14}$ 82—84°, n_D^{25} 1.4307) (Found: C 52.9; H 7.9; OCH_3 38.9. Calc. for $C_5H_6O_3(OCH_3)_2$ (160.2): C 52.5; H 7.6; OCH_3 38.7).

The following day the refractive index of the product had risen to 1.4323. Redistillation of 1.23 g gave 1.02 g of the original product (colorless liquid, b.p.$_{14}$ 82—83°, n_D^{25} 1.4306). Apparently V, like the corresponding dihydrofuran III, polymerizes on standing.

Reaction of liquid dimethoxydihydrofurfural diacetate with sodium methoxide. A portion (15.0 g) of the mother liquor from the isolation of I was treated with sodium methoxide in methanol and the red reaction mixture worked up as described for the preparation of III. The yield was 0.82 g (9 %) of III (b.p.$_{11}$ 93—94°, n_D^{25} 1.4450) (Found: C 53.0; H 6.6; OCH_3 39.2).

1. Clauson-Kaas, N., and Fakstorp, J. *Acta Chem. Scand.* **1** (1947) 415.

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Fixation of Nitrogen by Nodules Excised from Illuminated and Darkened Pea Plants

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In the symbiotic nitrogen fixing system of leguminous plant and root nodule bacteria, the photosynthesizing plant must translocate substrates to the nodule to support its general respiration and growth, to supply energy for nitrogen fixation, and to accept the fixed nitrogen. There is little information to indicate how rapidly these substrates are exhausted after photosynthesis ceases. Lindstrom, Newton and Wilson [1] have demonstrated a progressive and marked decrease in nitrogen fixation by red clover plants when they are darkened. Virtanen [2] has reported the conversion of leghaemoglobin to a green pigment in the nodules of plants kept in the dark, and found that such a conversion is connected with the inactivation of the nitrogen fixing system. The present experiments show that there is a striking and rapid decay in the nitrogen fixing capacity of nodules on darkened plants.

Torstai peas were planted in a sand substrate on June 30, were inoculated with *Rhizobium leguminosarum* strain H-43, and were furnished a nitrogen-free nutrient solution during their period of growth in a greenhouse. The plants, in early flower, were approximately 6 weeks old at the time of the experiments, and they were green, vigorous, and well nodulated. The nodules had red centers indicative of the presence of abundant leghaemoglobin.

At harvest the sand was rinsed from the roots, and the nodules were removed from the roots and placed immediately in a small respirometer vessel. The vessel on a Warburg manometer was evacuated [3], flushed twice with O_2 and reevacuated; a mixture

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of N_2 and O_2 to give a pN_2 of 0.1 atmosphere and a pO_2 of 0.2 atmosphere was added followed by argon to atmospheric pressure. The N_2 contained 60 atom % ^{15}N excess. The process of preparing the nodules and gassing them required from 16 to 33 minutes. The nodules, after being shaken in the respirometer for 2 hours, were ground with a mortar in 1.0 *N* HCl, and centrifuged. The sedimented material was discarded and only the acid-soluble portion of the nodules was analyzed (Table 1).

When the experiment was initiated, some of the pots of peas were placed in a large dark room, and the others remained in the greenhouse where they received unsupplemented summer daylight. The experiment was started at 12 noon on August 9, a bright day. After 24 hours in the dark some of the plants were returned to the greenhouse. August 10 and 11 were dull, rainy days, but the morning of August 12 again was sunny.

Although there was but one sample at the initial harvest, it is notable that on this day, when the plants were exposed to high light intensity, their nodules gave the highest fixation of N_2. Fixation by nodules from the lighted plants was much less at the 8 o'clock harvest August 10 than initially; fixation improved somewhat by noon but decreased again by noon August 11. At noon August 12, a bright day, the nodules maintained approximately the same level of fixation as on the previous day.

One sample from the darkened plants retained considerable nitrogen fixing capacity at 8 o'clock August 10, but the amount of nitrogen fixed by nodules harvested from darkened plants at 12 o'clock was very low. The ability to fix nitrogen continued to decay for the next 2 days. The plants returned to the greenhouse after 24 hours in the dark recovered their nitrogen fixing capacity only partially.

No gross change in the pigment of the nodules from the darkened compared to the illuminated plants was apparent after 24 hours, and only a slight decrease in pigment of the darkened plants occurred in 48 hours. At the final harvest (72 hours) the nodules of the darkened plants remained firm but were distinctly green inside

Table 1. Influence of illuminating and darkening pea plants on fixation of nitrogen by their excised nodules. All data are given as atom% ^{15}N excess in the portion of the ground nodules soluble in 1.0 N HCl.

Time of harvest	Treatment of plants	Atom % ^{15}N excess in nodule extract **
12 : 00, Aug. 9	initial control sample	0.627
8 : 00, » 10	greenhouse light * 20 hours	0.220, 0.213
8 : 00, » 10	darkened 20 hours	0.033, 0.210
12 : 00, » 10	greenhouse light 24 hours	0.417, 0.517, 0.520
12 : 00, » 10	darkened 24 hours	0.032, 0.022, 0.019
12 : 00, Aug. 11	greenhouse light 48 hours	0.389, 0.396
12 : 00, » 11	darkened 48 hours	0.012, 0.022
12 : 00, » 11	darkened 24 hours, returned to greenhouse 24 hours	0.233, 0.279
12 : 00, » 12	greenhouse light 72 hours	0.368, 0.398
12 : 00, » 12	darkened 72 hours	0.005, 0.007
12 : 00, » 12	darkened 24 hours, returned to greenhouse 48 hours	0.203, 0.126

* The normal daylight in the greenhouse was not supplemented with artificial illumination. Nights are still comparatively light about August 10 in Helsinki, but the morning in question was cloudy and rainy why light intensity was low. The poor effectivity of the nodules probably depends on this.

** We wish to thank Mr. Wayne E. Magee and Mr. Michael K. Bach for the analysis of the samples for ^{15}N.

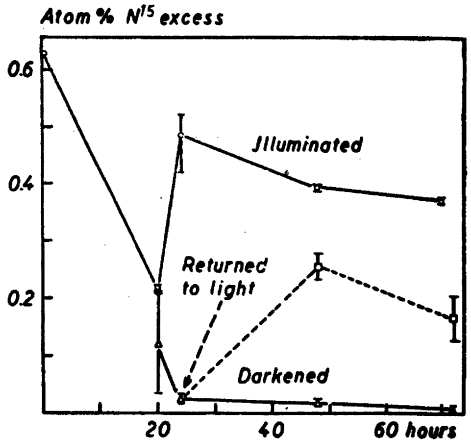

Fig. 1. Influence of illumination of pea plants on fixation by their nodules when exised. Vertical lines define the extreme values of replicate samples.

and exhibited a marked decrease in leghaemoglobin. The nodules from the illuminated plants were indistinguishable from those of the darkened plants which had been returned to light.

It seems most likely that the rapid decay of the nitrogen fixing capacity of nodules from plants kept in the dark for 24 h for the most part arises from depletion (and their rapid recovery in the light from renewal) of substrates furnished normally by the photosynthesizing plant, but partly also from the decrease in leghaemoglobin in the nodules. Virtanen [2] has shown that the oxydation of this pigment to a green one checks N_2-fixation, and a recent investigation by Virtanen and Berg has shown that a smaller part of leghaemoglobin is destroyed even during 24 h in darkness. Thus it can be understood that the transfer of plants to light causes a partial recovery of the effectivity of the root nodules, but far from the whole of it.

Whatever the basis for the observed response may be, the data clearly show that the nodules from pea plants kept in the dark have far less capacity for fixing N_2 than nodules from illuminated plants, and that the lost activity can be partially recovered by returning the plants to the light. It would be of interest to employ the isotopic method to determine in greater detail the rate of decay and recovery of nitrogen fixing capacity, the time required before the injury to the nitrogen fixing capacity becomes irreversible, and the influence of light intensity on the fixation process.

1. Lindstrom, E. S., Newton, J. W. and Wilson, P. W. *Proc. Natl. Acad. Sci., U. S.* **38** (1952) 392.
2. Virtanen, A. I. *Nature* **155** (1945) 747.
3. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. *Manometric Techniques and Tissue Metabolism*, Burgess Publishing Co., Minneapolis (1949), p. 44, 45.

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Estimation of Keto Acids in Plants

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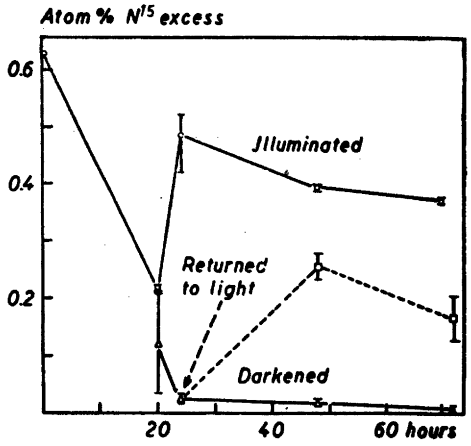
In the study of keto acids the paper chromatography of their 2,4-dinitrophenylhydrazones has been used. As the hydrazones of keto acids form multiple spots on the chromatogram, thus making the identification of the spots uncertain, unknown keto acids are difficult to discover. In order to elucidate unknown acid hydrazones in urine, Kulonen [1] reduced the hydrazones to the corresponding amino acids with aluminium amalgam according to the old method reported by Fischer [2], and investigated the amino acids by paper chromatography. Later Towers, Thompson, and Steward [3] used a similar method, the reduction being made catalytically. We have also developed a modification of the procedure which has already given some interesting results regarding keto acids in plants [4,5]. An account of it is given in the present paper. In our method the reduction is achieved by tin in an alcoholic hydrochloric acid solution, the yield of hydrogenolysis being comparatively good. The purification of the acid hydrazones obtained from the plant material was made according to Virtanen *et al.*[6]

Procedure. 1. *Preparation of the plant extract.* The plant material (usually about 100 g fresh wt.) was homogenized 3 times in a



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tungstic acid solution prepared just before use by mixing water, 10 % sodium tungstate, and 2/3 *N* sulphuric acid in the proportion 60:20:20 (v/v) according to Cavallini and Frontali [7]. The homogenate was filtered through a cheese cloth, the filtrates were combined and refiltered, this time through filter paper. The proportion between plant material and total extracting solution was in most cases 1:5 (w/v).

2. *Reaction with dinitrophenyl hydrazin.* The combined extracts were mixed with a 1 % solution of 2,4-dinitrophenyl hydrazine in 5 *N* H_2SO_4 (1 ml/g fresh wt. of plant material, Virtanen *et al.*[6]), and the reaction mixture was allowed to stand at room temperature for about 1 h. If a heavy precipitate was formed during the reaction the mixture was filtered once more.

3. *Extraction.* The reaction mixture was extracted according to Virtanen *et al.*[6] with ethyl acetate, and the ethyl acetate solution was extracted with 10 % Na_2CO_3. The combined soda phases were then acidified to about pH = 1 with 3 *N* H_2SO_4 and extracted with ethyl acetate. By this treatment the keto acid hydrazones could be separated from most of the amino acids and neutral carbonyl compounds in the original plant extract.

4. *Hydrogenolysis.* The final ethyl acetate extract was evaporated *in vacuo*, the residue was dissolved in 95 % ethanol and an aliquot (usually 2/3 or 4/5 of the solution, 1/3—1/5 being used as a control; see steps 6 and 7) was hydrogenated with tin by passing gaseous hydrochloric acid through the solution until a strong evolution of hydrogen gas started. Previously prepared HCl-ethanol was used in some cases. The vessel used in the hydrogenation was kept in an ice-water bath. The reaction time used varied from 12—20 h.

5. *Precipitation of the tin.* The reaction mixture was filtered, diluted with water, and the tin-ions were precipitated with hydrogen sulphide. The precipitate was then filtered off and the filtrate taken to dryness *in vacuo*.

6. *Purification with ion-exchange.* The residue was dissolved in water and passed through an Amberlite IR-120 column (25 × 1.3 cm). After washing the column with water the amino acids formed during the hydrogenolysis were displaced with 1 *N* NH_3. The amino acid solution which often had a brown colour was concentrated *in vacuo* or on a waterbath. The colour did not seriously interfere with the subsequent paper chromatography. The remaining non-hydrogenated keto acid hydrazones from step 4 were treated with Amberlite IR-120 in a similar way.

7. *Paper chromatography.* The amino acids formed were identified by two-dimensional chromatography using Whatman No. 4 paper and butanol-acetic acid and phenol + NH_3 as solvents. A corresponding amount of the similarly treated non-hydrogenated keto acid hydrazones was chromatographed as a control. It never contained more than traces of some amino acids present as contaminants.

Quantitative considerations. As to the quantitativity of the method we have obtained the following yields when treating known amounts of keto acids in accordance with the directions above: pyruvic acid 78 %, α-keto-butyric acid (impure prep.) 32 %, oxalacetic acid 61 %, and α-ketoglutaric acid 43 %. The values are approximate obtained by comparing the spots of the amino acids formed with spots of the corresponding amino acids in different concentrations. By starting directly from the synthetic hydrazones the yield for α-keto-butyric acid rises to about 60 %. Towers, Thompson, and Steward [3], using catalytical hydrogenation, got a yield of 65.3 % with pyruvic acid hydrazone, 97 % with glyoxylic acid hydrazone, but only 33.0 % and 36 % with the hydrazones of oxalacetic and α-ketoglutaric acid, respectively. Kulonen [1] in turn reports that the method of Fischer which he used for reduction is very unsatisfactory for the investigation of α-ketoglutaric acid, or of other dicarboxylic keto acids. The method for reduction used by us has, as mentioned above, given relatively good results both with ketodicarboxylic acids and keto-monocarboxylic acids. This has made the discovery of several new ketodicarboxylic acids possible [4,5]. The above mentioned values show that quantitative considerations are possible only if the yield of the hydrazone hydrogenolysis is known for each keto acid in question, the actual amounts may thus be 30—70 % higher than the amounts found on the chromatograms, in some cases possibly even higher. Our experiments in agreement with those of Towers, Thompson, and Steward [3] have also shown that 2,4-dinitrophenylhydrazone + ascorbic acid or dehydroascorbic acid does not give rise to noticeable amounts of amino acids during the hydrogenation procedure. The ninhydrin spots on chromatograms made from plant extracts by this method accordingly do not contain compounds derived from the above-mentioned substances.

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New α-Keto Acids in Green Plants. II. β-Hydroxy- and γ-Hydroxy-α-Ketobutyric Acid in Cowberries

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Using the method mentioned in our earlier paper [1] and in detail described in a later paper [2] we have identified a couple of new α-keto acids in some berries and green plants.

In berries of cowberry *(Vaccinium vitis idaea)* we identified the following α-keto acids (Fig. 1) (in brackets the amino acids formed by hydrogenolysis of the 2,4-dinitrophenylhydrazones of the corresponding keto acids): oxalacetic acid (aspartic acid), α-ketoglutaric acid (glutamic acid), hydroxypyruvic acid (serine), glyoxylic acid (glycine), γ-hydroxy-α-ketobutyric acid (homoserine), β-hydroxy-α-ketobutyric acid (threonine), and pyruvic acid (alanine). β- and γ-hydroxy-α-ketobutyric acid are new α-keto acids which have not earlier been found in any organism. The relatively intense spots T and Y represent unknown amino acids, and the corresponding keto acids are therefore also unknown.

If we assume that about 60 % of the keto acids [2] found in fresh cowberries (10.2 % dry substance, 0.93 % tot. N of dry subst., 10.5 % sol. N of tot. N) are reduced to amino acids when our method

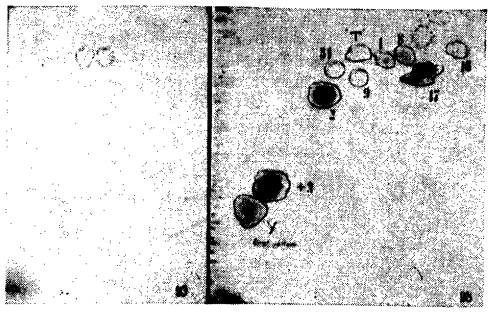


Fig. 1. Berries of Vaccinium vitis idaea. Left: Two-dimensional paper chromatogram (phenol-NH_3 and butanol-acetic acid) of the non-reduced 2,4-dinitrophenylhydrazones. Practically no spots of amino acids. Right: Two-dimensional paper chromatogram of the reduced hydrazones. 1 = gly, 2 = ala, 8 = ser, 9 = threo, 16 = asp, 17 = glu, 51 = homoser, T and Y = unknown amino acids. + 3 = valine added.

is used, the amount of different keto acids in 100 g of fresh cowberries are roughly the following: oxalacetic acid 25 μg, α-ketoglutaric acid 220 μg, pyruvic acid 130 μg, hydroxypyruvic acid 40 μg, glyoxylic acid 30 μg, γ-hydroxy-α-ketobutyric acid 20 μg, and β-hydroxy-α-ketobutyric acid 10 μg. As found by Alfthan and Virtanen [2] the 60 % yield which has been the basis for these calculations does not hold good for all amino acids. As, however, only yields

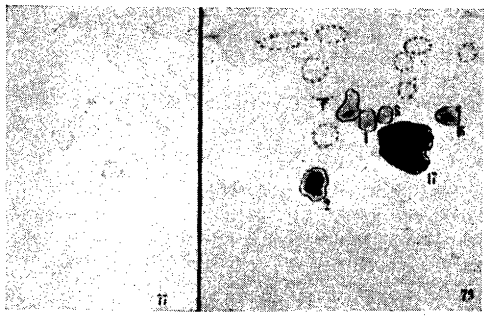


Fig. 2. Berries of Oxycoccus quadripetalus. Left: Two-dimensional paper chromatogram of the non-reduced 2,4-dinitrophenylhydrazones. Practically no spots of amino acids. Right: Two-dimensional paper chromatogram of the reduced hydrazones. Note the very strong spot of 17.

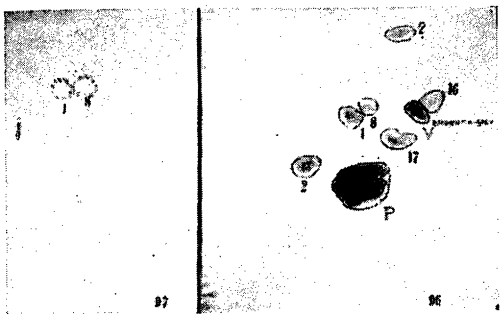


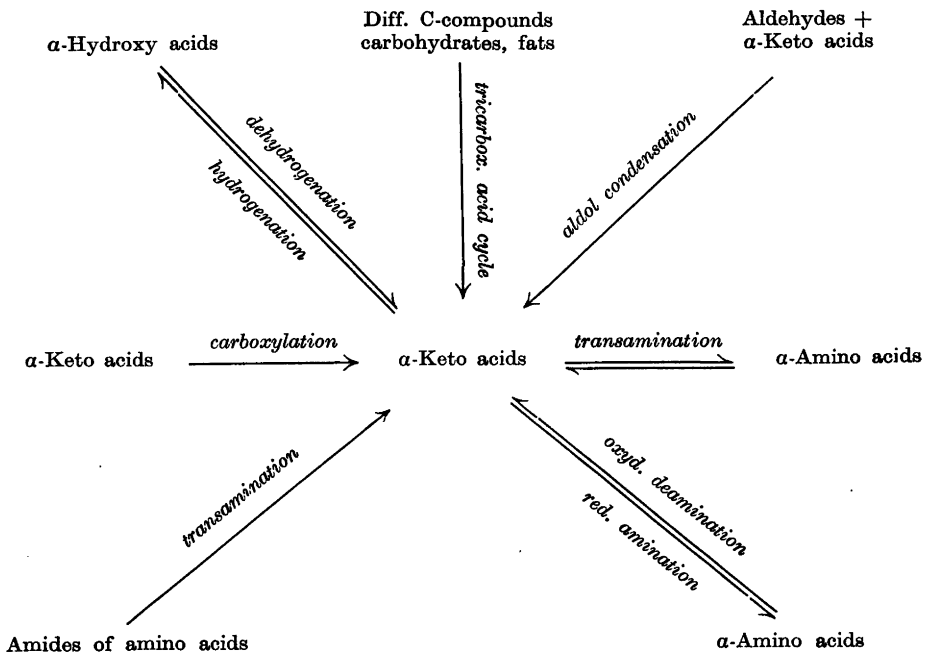
Fig. 3. Phyllitis scolopendrium. Left: Two-dimensional paper chromatogram of the non-reduced 2,4-dinitrophenylhydrazones. Practically no spots of amino acids, except extremely weak spots of 1 and 8. Right: Two-dimensional paper chromatogram of the reduced hydrazones. In addition to 1, 2, 8, 1ℓ, 17 the unknown amino acids V and P.

of a few amino acids formed from keto acids are known we have used the above mean yield.

In berries of mossberry *(Oxycoccus quadripetalus)* the keto acids are qualitatively nearly the same as in cowberry (Fig. 2). The keto acid corresponding to threonine was not, however, to be found in cowberry, at least not in the concentration used. The keto acid corresponding to the unknown amino acid Y was also lacking. The great amount of α-ketoglutaric acid in mossberry is especially remarkable.

Using the same basis for calculation as before the amount of different keto acids in mossberry (100 g of fresh berries) are roughly the following: oxalacetic acid 50 μg, α-ketoglutaric acid 2 700 μg, pyruvic acid 130 μg, hydroxypyruvic acid 25 μg, and glyoxylic acid 35 μg.

In a fern *(Phyllitis scolopendrium)* Virtanen and Berg [3] have found very large amounts of an unknown acidic OH-containing amino acid P, and a smaller amount of another amino acid V. (*cf.* Fig. 3). These authors are going to give a more detailed account of the isolation, structure, and properties of these new amino acids. Our keto acid determinations show (Fig. 3) that the keto acid corresponding to the amino acid P occurs very abundantly in this plant as compared with other keto acids, as, according to Virtanen and Berg, the amount of this amino acid is also by far the greatest when compared with other free



amino acids. The amount of the keto acid corresponding to the amino acid V is much smaller as is also that of the amino acid V in comparison with other free amino acids.

It is often to be noted that a great amount of an amino acid corresponds to a great amount of keto acid, at least when uncommon free amino acids are in question. This gives support to the conception that these amino acids may be formed from the corresponding keto acids *via* transamination.

The occurrence of a number of different α-keto acids corresponding to different amino acids in plants is due to the fact that these keto acids are either precursors to, or deamination products of, the corresponding amino acids. If deamination occurs *via* transamination the keto acids are both deamination products and precursors because the reaction is reversible, the equilibrium depending on the concentration of keto and amino acids participating in the transamination. Also the keto acid formed *via* oxidative deamination can be transformed into the corresponding amino acid *via* transamination. From amino acids which are formed from other amino acids *via* specific reactions (as *e. g.* homoserine from aspartic acid, threonine from homoserine *etc.*) the corresponding keto acid can be formed *via* deamination, and from this again *via* transamination the corresponding amino acid. The following scheme presents different possible pathways for the formation of α-keto acids in organisms and also for the formation of amino acids and amides, respectively, from α-keto acids.

The appearance of numerous widely different α-keto acids in plants gives further proof for the importance of these acids in plants.

We are very grateful to Dr H. Huber, Basel, for *Phyllitis scolopendrium*.

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Effects of Ions on the Activity of Enzyme Systems

W. J. Rutter and B. Rolander

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm

The observed activity of many enzyme systems is a function of the ion population of the medium. Recent kinetic studies from Theorell's laboratory [1-3] have emphasized the role of anions in determining the rates of certain enzyme reactions. In an effort to determine the generality and the order of magnitude of these ion effects, several additional systems have been studied. The activity of the glucose-6-phosphate dehydrogenase system was markedly (as much as six fold) stimulated by low concentrations of various ions, while at higher concentration an inhibition was observed *. The magnitude of the stimulation and the onset of inhibition depended upon an interrelationship between the concentration of the substrate, TPN, and the concentration of the specific ions present. The older experiments [4,5], which indicated only a strong inhibition by phosphate are probably a result of a very low effective concentration of the substrate. Studies with various salts indicated that the observed effects were primarily determined by the particular anion species present. The order of magnitude of stimulation is roughly

$$F^- > PO_4^{=} > Cl^- > Br^- > SO_4^{=} > I^- > SCN^-.$$

Large anions like glycyl-glycine and TPN are poor activators, but the substrate, glucose-6-phosphate is an effective "anion activator". The differences in stimulation produced by various cations was generally related to the magnitude of contribution to the ionic strength of the medium. The overall results favor the concept of a non-specific ionic effect rather than an interpretation which involves a heavy metal requirement [6,7].

The observed activity of certain coupled systems, for example the Zwischenferment-old yellow enzyme system and the succinate oxidase system is also related to the kind and concentration of anions present.

These effects have been interpreted according to the general principles of solvation of chemical reactions.

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The Variation in the Structure of Water on Gelation

Bertil Jacobson

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X-ray diffraction patterns of various gels were studied at 20° C in a Guinier camera employing Cu$K\alpha$ radiation. The intensity curves, obtained in the usual way from photometer determinations of film densities, were corrected for polarization and absorption.

Dilute gels (0.3—2.0 %) of certain polyelectrolytes (desoxyribonucleic acid, carboxymethylcellulose) and of a low molecular weight substance (dibenzoylcystin) give similar diffraction patterns with small but characteristic differences from that of pure water at the same temperature. The major peak at $(\sin\Theta) / \lambda = 0.16$ Å^{-1} shifts toward smaller angles and the height of the minor peak at 0.23 Å^{-1} increases relative to the properties of pure water. There also seems to be a tendency for the minor peak to shift toward larger angles.

From diffraction data it is known that the structure of water changes with temperature [1]. With decreasing temperature the tendency of a water molecule to bond itself tetrahedrically to four other neighboring molecules increases. This is observed as a shift in the major peak towards smaller angles and of the minor peak towards larger angles and in an increase in the height of the minor peak.

Thus the changes on gelation are qualitatively similar to those obtained in pure water when the temperature is decreased; that is, the gel-forming substances produce a decrease in the "structural temperature" of the water. The effect is opposite to that found with electrolytes which are known to increase the "structural temperature" [2].

The experimental results confirm the Forslind theory of gelation [3] according to which the thermal vibrations in the water lattice are reduced through a specific coupling to the non-aqueous component of the gel. The stabilization of the water is the result of a structural similarity between the ideal, four-coordinated water lattice and the positions of the hydrogen bond forming atoms on the surface of the non-aqueous phase.

An identical theory was developed in order to explain certain physico-chemical properties of desoxyribonucleic acid solutions [4]. Dielectric properties, viscosity and proton magnetic resonance data were explained through assuming the existence of large hydration shells with a higher degree of water lattice order than that prevailing in pure water. The present X-ray diffraction data are in agreement with the theory that certain asymmetric macromolecules can stabilize the water lattice over distances of some hundred Å.

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Carboxymethyl Chitin, a New Substance Suitable for the Determination of Chitinase Activity

Eskil Hultin

Institutet för organisk kemi och biokemi, University, Stockholm, Sweden

The chitinases have not hitherto been investigated as extensively as could be expected for enzymes that break down such a common substance as chitin. This substance occurs in large quantities both in several animals (*e. g.*, crustaceans, insects and molluscs) and in various plants (*e. g.*, fungi). Chitinases are also known from various sources, *e. g.*, the "liver" of snails, insect larvae, sperm, a variety of bacteria, molds, and almonds.

The following substrates have been used for the determination of chitinase activity: native chitin, regenerated chitin (precipitated by diluting a solution of chitin in cold concentrated hydrochloric acid), chitosan (deacetylated chitin), chitotriose and chitobiose. As chitin is insoluble in water, it is not suitable as a substrate for the determination of enzymic activity. Chitosan is better for it is soluble in buffer solutions with pH less than about 6.5. In more alkaline solutions it is insoluble and hence not suitable. It is surely not the same enzyme [1,2] that attacks chitin and its oligosaccharides (*cf.* amylase and maltase).

A new chitin derivate, not described previously, carboxymethyl chitin, is suggested now as a substrate for the determination of chitinase activity. It has not the disadvantages of the substances used previously.

The preparation of carboxymethyl chitin. Raw chitin is prepared in the well-known way, *e. g.*, by treating shells of crustaceans with a dilute acid. The chitin is dissolved in supersaturated cold hydrochloric acid and precipitated by adding water or alcohol. The regenerated chitin is heated on a water bath with chloroacetic acid and a strong solution of sodium hydroxide in the way well-known for the preparation of carboxymethyl cellulose. Native chitin does not react with sodium hydroxide and chloroacetic acid under these conditions. The reaction product is dissolved in water and after neutralization the sodium salt of carboxymethyl chitin is precipitated by adding alcohol.

The determination of chitinase activity. A solution of carboxymethyl chitin is rather viscous, and so it is possible to run the determination of chitinase activity viscosimetrically in the well-known way [3]. This is advantageous

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because viscosimetric methods usually are considerably more sensitive than other methods. As the substrate is a polyvalent acid, the ionic strength in the reaction mixture must be kept constant (*e. g.*, 0.1), and the concentration of multivalent cations should be as low as possible. Addition of cysteine or other compounds containing sulphydryl groups causes a steady decrease in the viscosity of carboxymethyl chitin solutions. However, this difficulty can be overcome — in the same way as in the case of carboxymethyl cellulose [4] — by adding a small amount of potassium ferricyanide.

Senju and Okimasu [5] have described the preparation of hydroxyethylchitin, which is probably a suitable substrate for chitinase determination but this has not yet been proved.

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The Zimmermann Method

Sven Arrhenius

Kemiska Avdelningen, Kungl. Veterinärhögskolan, Stockholm, Sweden

This method [1] is the most frequently used in steroid analysis. The sample is dissolved in ethanol, made alkaline with KOH, and stained with *m*-dinitrobenzene. If the sample contains steroids with a ketogroup in position 17, the extinction curve will show a flat maximum at 5 300 Å. In their absence the curve will only slope towards longer wave-lengths. The reactions causing the staining are not known. None of the many variants of the method is accurate. One source of error is the oxidation of ethanol by the nitro compounds. The aldehyde thus formed polymerises in alkaline solution.

m-Dinitrobenzene is soluble in most organic solvents. As no staining will occur unless $pOH < 2$, the only suitable solvents for the reaction are the simplest alcohols. The tertiary butanol is the only alcohol not oxidised by *m*-dinitrobenzene. It was used for an investigation of the Zimmermann reaction. The following results were obtained:

1. Alcaline solutions of pure *m*-dinitrobenzene in tert. butanol have the same extinction curves as those described for stained 17-ketosteroids.

2. The intensity of the colour depends mainly on the concentration of OH^-. It explains why the colours disappears after a couple of days.

3. Some carbonyl compounds as acetaldehyde, acetone, *cyclo*hexanone, and 17-ketosteroids increase the extinction, and shift the absorbtion maximum over to longer wavelengths (5 600 Å). Easily enolised ketones and formaldehyde have no or only slight influence on the colour.

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The Incorporation *in vivo* of Amino Acids into Subfractions of Cytoplasmic Particles

Tore Hultin

The Wenner-Gren Institute, Stockholm, Sweden

It has been shown previously [1,2] that in chick liver the incorporation *in vivo* of labeled amino acids is particularly high in the submicroscopic particulate components of the cytoplasm.

C^{14}-glycine, N^{15}-glycine, or N^{15}-DL-alanine were injected intravenously into chicks or rats. The animals were killed 1—5 minutes after the administration of the isotope. Fractions of large and small submicroscopic particles were prepared from homogenates of the livers by differential centrifugation. (After removal of the mitochondrial fractions, the homogenates were centrifuged for 20 minutes at 20 000 g, and subsequently for 40 minutes at 105 000 g). After several washings, the particles were repeatedly treated at 0° C with 0.2 *M* $NaHCO_3$, pH 8.4, and with 0.6 % desoxycholate, pH 8.4. The cholate extracts were further fractionated by means of ethanol at low temperatures [3].

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Results. A very rapid increase of "glycogen"/mg of dry cells was found, reaching its maximum at 30 minutes (Table 1). This indicates that the "glycogen" synthesis precedes the synthesis of the bulk of the cell-material.

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The technique permits the estimation of the total amounts of primary glycosides, secondary glycosides and aglycones. The filter capacity of a 10 cm long column is 2—2 000 μg, but the method can be scaled up for larger amounts. The mean recovery is 95—98 %, and the experimental error of a double determination is $\pm$ 2.59 %.

The separation may be regarded as a particular application of ion exchange resins based on the swelling properties of their network structure when the alteration of the size of pore spaces is carried out by suitable pretreatment and elution. This assumption may be supported by the fact that (1) the separation can be achieved with the resin in the free-acid form as well as in the sodium form, (2) the glycosides retarded could be eluted neither with acids nor alkalis, and (3) the eluting power of the same organic solvent seems to be altered by changing the prewashing agent.

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Methods for Studying Nucleotide Metabolism in Synchronized Cultures of Protozoa

Paul Plesner

Institute of Cytophysiology, University of Copenhagen, Denmark

In an attempt to study the nucleotide metabolism in different phases of the cell cyclus, and to correlate the pattern of the *in vivo* findings with the *in vitro* activity of some of the cellular enzymes, the technique of Zeuthen and Scherbaum [1] has been employed to synchronize cultures of *Tetrahymena pyriformis*. An apparatus, that can accommodate 10 cultures of 150 ml each and expose them to the temperature shocks required, has been constructed. The cells are grown on a proteose-peptone-salt medium or on this medium after treating it with 5 grams of norite per liter and fortifying it with Kidder's vitamin B group [2] and 15 mg per liter of guanylic and uridylic acids each ("semisynthetic medium"). Norite absorbs all pyrimidines, purines and all their ribosides and ribotides.

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Since the sugar moiety of the cardiac glycosides consists of highly specific desoxysugars, a possibility is afforded of detecting glycosides by utilizing the specificity of the sugar component. The data recorded show that desoxymethylpentoses, *i. e.* the specific sugar compound occurring only in the cardiac glycosides, give a reaction with orcinol which may be used for the quantitative determination of these glycosides. Although a great variety of carbohydrates react with orcinol, the test, in this modified form, seems to be highly specific for desoxymethylpentoses. This permits the detection of minute amounts of cardiac glycosides even in several thousandfold excess of common sugars. All the primary and secondary cardiac glycosides of digitalis give the reaction, as do k-strophantin and oleandrin. Ouabain and the squill glycosides, both containing a methylpentose, do not react, nor do other methylpentoses or desoxypentoses. Aglycones, saponins and other steroid derivatives do not interfere with the reaction. A further advantage of the test is that all the glycosides react in relation to their theoretical sugar content. The method may be used for the quantitative determination of desoxymethylpentose in amounts of 0.5—60 μg; this amount corresponds to about 1—100 μg of glycosides. The experimental error of a double determination is $\pm$ 1.51 %.

On the Effect of Substituted Barbiturates * on Mitochondrial Respiration

Olaf Jalling**, Olov Lindberg and Lars Ernster

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Substituted barbiturates have long been known to affect the cellular respiratory system. In 1949, Eiler and McEwen [1], working with brain homogenates, showed that the oxidation of pyruvate and fumarate could be blocked by pentobarbital while that of succinate was unaffected. Later investigations, performed on isolated mitochondria, did not lead, however, to a generally valid interpretation of these findings as to the mode of barbiturate action. Brody and Bain [2] reported a decrease of the phosphorylation/oxidation ratio and attempted to interpret the barbiturate action in terms of an uncoupling effect rather than a respiratory inhibition. This view, however, could not be shared by Johnson and Quastel [3]. These authors found, as an effect of barbiturates, a decreased capacity of brain mitochondria to activate acetate, but came to the conclusion that this effect might be a secondary one.

From the above mentioned results of Eiler and McEwen it may be envisaged that the effect of barbiturates must be of a rather fundamental character and may therefore also be studied in a system metabolically better defined than brain, *e. g.* liver mitochondria. In experiments with this system we have found that amytal, in a concentration of $1.8 \cdot 10^{-3}$ *M* totally inhibits the oxidation of a series of substrates (Table 1); a clearly marked exception is formed by succinate, the oxidation of which is not at all influenced by amytal. This finding — which is consistent with, and an extension of, the early results by Eiler and McEwen — indicates that the action of barbiturates is concerned with the transfer of hydrogen *via* the pyridine nucleotides.

In some preliminary experiments we were also able to obtain an indication of a correlation between narcotic power and inhibition of respiration. This is shown in Table 2 where

* pentobarbital = 5-ethyl-5-(1-methylbutyl)barbiturate; amytal = 5-ethyl-5-*iso*amylbarbiturate; hydroxy-amytal = 5-ethyl-5(3-hydroxy-*iso*amyl)barbiturate.

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the inhibitory effects of identical concentrations of a narcotic (amytal), a sedative (pentobarbital) and a pharmacologically inactive barbiturate (hydroxyamytal) are compared.

The results suggest that pharmacologically active barbiturates exert an inhibitory effect on hydrogen transfer *via* pyridine nucleotides with a general inhibition of cell respiration as a consequence. The fact that this effect is restricted to the central nervous system *in vivo* may be interpreted as indicating that a limitation of the respiratory capacity is expressed more primarily in this tissue than in other organs.

Table 1. Effect of amytal on the oxidation of different substrates in rat liver mitochondria.

Substrate	Respiration (microatoms oxygen)	
	without amytal	*with* 1.8 m*M* amytal
L-glutamate	26.2	0
pyruvate	9.5	0
citrate	11.7	0
α-ketoglutarate	22.0	3.5
succinate	26.2	23.4
fumarate	9.4	0
L-malate	8.5	0
DL-β-hydroxybutyrate	8.5	0

Each Warburg vessel contained: rat liver mitochondria (prepared in 0.25 *M* sucrose — 0.01 *M* versene), 1/12 liver; substrate, 30 micromoles; adenylic acid, 4.3 micromoles; orthophosphate, 40 micromoles; glucose, 47 micromoles; KCl, 270 micromoles; Mg^{++}, 7.5 micromoles; yeast hexokinase (prepared according to Berger *et al.*, "Step 5"), 0.05 ml. pH 7.5. Final volume, 2.0 ml. Temp. 30° C. Gas phase, air. Time of incubation, 25 min.

Table 2. Inhibition of mitochondrial respiration by different derivatives of barbiturate

Barbiturate $M \cdot 10^3$	amytal	pentobarbital	hydroxyamytal
	percent inhibition of respiration		
0.9	67	16	0
1.8	100	44	7

Substrate, glutamate. Experimental conditions as in Table 1.

1. Eiler, J. J. and McEwen, W. K. *Arch. Biochem.* **20** (1949) 163.
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Yield of Oxidative Phosphorylation in the Succinic Oxidase System

Hans Löw, Lars Ernster and Olov Lindberg

Wenner-Gren's Institute, Stockholm, Sweden

We have recently shown[1] that Ca^{++}-treated rat liver mitochondria catalyze the one-step oxidation of succinate to fumarate. The addition of adenosine triphosphate and Mn^{++} to this system results in a coupled phosphorylation. At the optimal concentration of Mn^{++}, and with increasing amounts of adenosine triphosphate, the phosphorylation / oxidation (P/O) ratio asymptotically approaches the value of 1.1.

Another way of obtaining a single-step oxidation of succinate is by means of substituted barbiturates which, as shown previously (Jalling *et al.*, *cf.* p. 198), are able to block further oxidation of the fumarate formed. Eiler and McEwen [2], using 5-ethyl-5-(1-methylbutyl)barbiturate as a blocking agent, obtained a P/O ratio of about 1.4 for the one-step oxidation of succinate in rat brain homogenates. In rat liver mitochondria treated with $1.8 \cdot 10^{-3}$ *M* 5-ethyl-5-*iso*amylbarbiturate (amytal) we have obtained about the same value, 1.42 (Table 1). There is thus a significant difference in the P/O ratio between the succinic oxidase systems obtained in the two different ways. Obviously, the Ca^{++}-treated system does not yield a maximum P/O ratio.

On the other hand the data in Table 1 show that the value obtained in the Ca^{++}-treated system can be lowered further by amytal. This indicates that amytal may be able to lower the P/O ratio in the succinic oxidase system, *i. e.* that not even the value of 1.42 may be regarded as uninfluenced. The possible effect of amytal on the phosphorylation coupled with succinate oxidation is a subject of further study.

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citrate	11.7	0
α-ketoglutarate	22.0	3.5
succinate	26.2	23.4
fumarate	9.4	0
L-malate	8.5	0
DL-β-hydroxybutyrate	8.5	0

Each Warburg vessel contained: rat liver mitochondria (prepared in 0.25 *M* sucrose — 0.01 *M* versene), 1/12 liver; substrate, 30 micromoles; adenylic acid, 4.3 micromoles; orthophosphate, 40 micromoles; glucose, 47 micromoles; KCl, 270 micromoles; Mg^{++}, 7.5 micromoles; yeast hexokinase (prepared according to Berger *et al.*, "Step 5"), 0.05 ml. pH 7.5. Final volume, 2.0 ml. Temp. 30° C. Gas phase, air. Time of incubation, 25 min.

Table 2. Inhibition of mitochondrial respiration by different derivatives of barbiturate

Barbiturate $M \cdot 10^3$	amytal	pentobarbital	hydroxyamytal
	percent inhibition of respiration		
0.9	67	16	0
1.8	100	44	7

Substrate, glutamate. Experimental conditions as in Table 1.

1. Eiler, J. J. and McEwen, W. K. *Arch. Biochem.* **20** (1949) 163.
2. Brody, T. M. and Bain, I. A. *Proc. Soc. Exptl. Biol. Med.* **77** (1951) 50.
3. Johnson, W. J. and Quastel, J. H. *J. Biol. Chem.* **205** (1953) 163.

Yield of Oxidative Phosphorylation in the Succinic Oxidase System

Hans Löw, Lars Ernster and Olov Lindberg

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We have recently shown[1] that Ca^{++}-treated rat liver mitochondria catalyze the one-step oxidation of succinate to fumarate. The addition of adenosine triphosphate and Mn^{++} to this system results in a coupled phosphorylation. At the optimal concentration of Mn^{++}, and with increasing amounts of adenosine triphosphate, the phosphorylation / oxidation (P/O) ratio asymptotically approaches the value of 1.1.

Another way of obtaining a single-step oxidation of succinate is by means of substituted barbiturates which, as shown previously (Jalling *et al.*, *cf.* p. 198), are able to block further oxidation of the fumarate formed. Eiler and McEwen [2], using 5-ethyl-5-(1-methylbutyl)barbiturate as a blocking agent, obtained a P/O ratio of about 1.4 for the one-step oxidation of succinate in rat brain homogenates. In rat liver mitochondria treated with $1.8 \cdot 10^{-3}$ *M* 5-ethyl-5-*iso*amylbarbiturate (amytal) we have obtained about the same value, 1.42 (Table 1). There is thus a significant difference in the P/O ratio between the succinic oxidase systems obtained in the two different ways. Obviously, the Ca^{++}-treated system does not yield a maximum P/O ratio.

On the other hand the data in Table 1 show that the value obtained in the Ca^{++}-treated system can be lowered further by amytal. This indicates that amytal may be able to lower the P/O ratio in the succinic oxidase system, *i. e.* that not even the value of 1.42 may be regarded as uninfluenced. The possible effect of amytal on the phosphorylation coupled with succinate oxidation is a subject of further study.

Table 1. The action of different agents on the P/O ratio in the succinic oxidase system.

Agent blocking fumarate oxidation	Additions after 5 min of pre-incubation with blocking agent	Respiration (μatoms oxygen)	Phosphorylation (μmoles phosphate)	P/O
—	succinate	16.6	27.6	1.66
amytal	succinate	16.2	23.0	1.42
Ca^{++}	succinate, ATP, Mn^{++}	16.9	19.0	1.12
amytal + Ca^{++}	succinate, ATP, Mn^{++}	16.8	15.3	0.91

Each Warburg vessel contained: rat liver mitochondria (prepared in 0.25 *M* sucrose — 0.01 *M* versene), 1/12 liver; adenylic acid, 4.3 μmoles; orthophosphate, 40 μmoles; glucose, 47 μmoles; KCl, 270 μmoles; Mg^{++}, 7.5 μmoles; yeast hexokinase (prepared according to Berger *et al.*, "Step 5"), 0.1 ml. Additions (where indicated): succinate, 30 μmoles; amytal, 3.6 μmoles; Ca^{++}, 1.15 μmoles; ATP, 1.5 μmoles; Mn^{++}, 1.5 μmoles. pH 7.5. Final volume, 2.0 ml. Temp 30° C. Gas phase, air. Time of incubation, 20 min.

1. Ernster, L., Lindberg, O. and Löw, H. *Nature (In press)*.
2. Eiler, J. J. and McEwen, W. K. *Arch. Biochem.* **20** (1949) 163.

The Action of 5-Ethyl-5-*iso*amylbarbiturate (Amytal) on the Oxidation of Reduced Diphosphopyridine Nucleotide (DPNH) in Rat Liver Mitochondria

Lars Ernster, Hans Löw and Olov Lindberg

Wenner-Gren's Institute, Stockholm, Sweden

Previously (Jalling *et al.*, *cf.* p. 198) it has been shown that $1.8 \cdot 10^{-3}$ *M* amytal completely blocks those mitochondrial oxidations which proceed *via* pyridine nucleotides. To study further the localization of this effect within the respiratory chain, DPNH, as generated by added alcohol dehydrogenase [1] and ethanol was used as a substrate for mitochondria. As seen in Table 1, the respiration in this system is, in contrast to the case of DPNH generated by the mitochondrial dehydrogenases, but partially — about 33 % — inhibited by $1.8 \cdot 10^{-3}$ *M* amytal. The accompanying phosphorylation is, on the other hand, almost completely inhibited.

This indicates that DPNH generated by "external" dehydrogenases is probably oxidized by two pathways, only one of which is sensitive to amytal. Both pathways differ markedly with respect to the extent of phosphorylation coupled to them. In the system studied, the amytal-insensitive pathway seems to be inaccessible to DPNH generated by mitochondrial dehydrogenases.

These findings suggest a possible similarity between the action of amytal and that of antimycin A on mitochondrial DPNH- and TPNH-cytochrome *c* reductase systems, as recently studied by Reif and Potter [2] and by Pressman and de Duve [3].

Table 1. The effect of amytal on the oxidation of DPNH in rat liver mitochondria.

	Without amytal	*With* 1.8 m*M* amytal
Respiration (μatoms oxygen)	9.6	6.4
Phosphorylation (μmoles phosphate)	13.1	2.2
P / O	1.37	0.34

Each Warburg vessel contained: rat liver mitochondria (prepared in 0.25 *M* sucrose — 0.01 *M* versene), 1/12 liver; adenylic acid, 4.3 μmoles; orthophosphate, 40 μmoles; glucose, 47 μmoles; cytochrome *c*, 0.0002 μmoles; KCl, 270 μmoles; Mg^{++}, 7.5 μmoles; yeast hexo-

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kinase (prepared according to Berger *et al.*, "Step 5"), 0.1 ml; alcohol dehydrogenase, 60 γ. Additions after 5 min. of preincubation with or without amytal: ethanol, 30 μmoles; DPN, 0.67 μmoles. pH 7.5. Final volume, 2.0 ml. Temp. 30° C. Gas phase, air. Time of incubation, 26 min.

1. The alcohol dehydrogenase was generously supplied by Dr. A. P. Nygaard, Biochemical Dept., Medical Nobel Institute, Stockholm.
2. Reif, A. E. and Potter, V. R. *Arch. Biochem. Biophys.* **48** (1954) 1.
3. Pressman, B. C. and de Duve, C. *Arch. intern. physiol.* **62** (1954) 306.

Trehalosemonophosphoric Acid, a Probable Intermediate in the Formation of Trehalose in Yeast

Majken Elander

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

In a recent note it has been shown by Leloir and Cabib [1] that trehalosemonophosphoric acid (THP) is formed from UDPG and G-6-P in extracts from yeast. Earlier Robison and Morgan [2] had found that this ester is normally produced during fermentation of glucose and fructose with dried yeast and zymin. Some years later Veibel [3] investigated the formation of THP during the fermentation of glucose with fresh brewers' yeast, incubated with toluene. He found that such a considerable amount of THP was formed that it could neither have been preformed in the yeast nor could it have arisen through phosphorylation of trehalose in the yeast itself. Considerably later it was shown that even formation of free trehalose occurs during fermentation of glucose with mazeration juice from brewers' yeast [4,5]. Elander and Myrbäck assumed that the disaccharide was formed through dephosphorylation of THP. Later the author has tried in vain to demonstrate THP in the same samples where trehalose was present, when the fermenting system was acetone dried bakers' yeast. The preformed trehalose of the yeast was removed by washing with phosphate buffer, pH 6.3. Coenzymes necessary for fermentation and probably also for the formation of trehalose were removed hereby, too, but were supplied through addition of boiled extract from air dried brewers' yeast which usually contains only small amounts of trehalose.

The reason of the failure to demonstrate THP in the presence of trehalose was not elucidated until close analysis of the different fractions of phosphate esters had been performed. The results will be published later in detail. Thus directly reducing esters, fructose, organic phosphate and the changes of these components at different times of hydrolysis have been determined. The optical rotations of the fractions were measured, and they were also subjected to paperchromatographic analysis. The analysis showed that one have to count with at least two hexosemonophosphoric esters which were unknown when Robison and Morgan studied the formation of THP. At this time they only had to deal with F-1,6-P, F-6-P and G-6-P, and the properties of these esters were well known. One of the new esters was G-1-P which has been shown by Cori, Colowick and Cori [6] and by Kiessling [7] to be an intermediate in the formation and degradation of glycogen in yeast. The other one has, according to the analysis, turned out to be F-1-P. Its occurrence in yeast is so far not known. The amount of the two phosphoric acids is quite considerable and they interfere with such determinations of THP as the optical rotation and the reducing power after hydrolysis. G-1-P as well as THP shows a high positive rotation. The rotation of F-1-P is negative. After acid hydrolysis G-1-P as well as THP becomes reducing. F-1-P, F-6-P and free fructose lose about 50 % of their reducing power when hydrolysed for 5 hours in 1 *N* H_2SO_4 at 100°. As the fructose esters formed a considerable part of the total esters, their decrease in reducing power will counteract the increase from THP and G-1-P.

After these investigations it was clear that a reliable determination of THP could only be performed if the concentration of G-1-P and F-1-P were known or if these esters could be removed from the fractions containing THP.

In a new series of experiments it was shown that G-1-P and F-1-P could be completely removed by precipitation with $BaAc_2$ and alcohol while the main part of THP was left in the trehalose fraction which only contained difficultly hydrolyzable organic phosphate. In fermenting mixtures THP is the only known ester with this property which is not reducing It could thus be determined as the difference between glucose equivalent of organic phosphate and the glucose equivalent of directly reducing substances. During the time of the experiments, 24 hours, the concentration of THP was rather constant while the concentration of trehalose after 4 hours reached a maximum. After that it was slowly broken

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down. When the concentration of trehalose was highest THP formed 21 % of trehalose. Details of the experiments will be published later.

The investigation is to be continued with paperchromatographic analysis and with attempts to isolate THP and F-1-P.

1. Leloir, L. F. and Cabib, E. J. *J. Am. Chem. Soc.* **75** (1953) 5445.
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3. Veibel, S. *Biochem. Z.* **239** (1931) 350; **252** (1932) 305.
4. Elander, M. and Myrbäck, K. *Arch. Biochem.* **21** (1949) 249.
5. Nilsson, R. and Alm, F. *Acta Chem. Scand.* **3** (1949) 213.
6. Cori, G. T., Colowick, S. P. and Cori, C. F. *J. Biol. Chem.* **123** (1938) 375.
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On the Intestinal Absorption of Phospholipids in the Rat

Rolf Blomstrand

Department of Physiological Chemistry, University of Lund, Lund, Sweden

Earlier works on phospholipids by Artom and Swanson[1] and Bloom *et al.*[2] have been extended.

The mechanism concerned with the absorption of phospholipids has been studied on the rat using phospholipids, labelled in the glycerol, fatty acid or phosphate portions of the molecule and the distribution of radioactivity in the collected lymph from the thoracic duct has been studied.

The experimental data demonstrate that phospholipids are hydrolyzed to a considerable extent during their absorption. Glyceride glycerol is utilized for the synthesis of lymph phospholipids and phospholipid glycerol for glycerides indicating an interconversion of these lipids during absorption.

1. Artom, C. and Swanson, M. *J. Biol. Chem.* **175** (1948) 871.
2. Bloom, B., Kiyasu, J. Y., Reinhardt, W. O. and Chaikoff, I. L. *Am. J. Physiol.* **177** (1954) 84.

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Studies on Antimetabolites

VI. Synthesis of D,L-"Neothyroxine", the β,β-Dimethyl Analogue of D,L-Thyroxine *

E. DUINTJER and Å. JÖNSSON

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The β,β-dimethyl analogue of D,L-thyroxine has been prepared by the reaction sequence shown in Fig. 1. A second series of reactions with the same goal was also carried out (Fig. 2) but had to be abandoned, as the hydantoins VII and VIII could not be hydrolysed to the corresponding 3,5-diiodothyroxine derivative.

The search for substances possessing antithyroid activity has lead to the preparation of a number of analogues of thyroxine. Among the compounds tested as thyroxine antagonists are aliphatic ethers of 3,5-diiodotyrosine, the sulphide analogue of thyroxine, 4-benzyloxy-3,5-diiodobenzoic acid and similar compounds, halogeno and nitro derivatives of diphenyl ether, thyronine and 4-(*p*-hydroxyphenoxy)benzoic acid [1], α-thyroxine and some derivatives of thyroxine in which the side chain had been modified by conversion of the carboxyl group into the methyl ketone [2]. Many of these compounds possessed some anti-thyroxine activity, but none of them appears to have been used clinically.

In continuation of our researches on the physiological effect of the introduction of a *gem*dimethyl group in various amino acids, hormones and other compounds of biochemical interest [3] we have now prepared the α,α-dimethyl analogue (XVI) of D,L-thyroxine, and it is the purpose of the present paper to describe the synthetic work. The results of the physiological testing of the compound will be reported elsewhere. For convenience the compound is henceforth called D,L-"*neo*thyroxine", thus indicating the analogy with *neo*pentyl derivatives.

The synthesis was started from D,L-"*neo*tyrosine" (I), the preparation of which was described in Part I of this series [3], and follows the same route, which was worked out by Burrows *et al.*[4] and by Chalmers *et al.*[5] for the preparation of thyroxine from tyrosine, with some alterations in experimental details.

* Part V. *Acta Chem. Scand.* 8 (1954) 1492.

R = CH3 C CH · COOC2H5 CH2 NHCOCH3
I
HONO2
II
(CH3CO)2O NaOH
IX
HONO2
X
C2H5OH SOCl2
XI
p-CH3 · C6H4 · SO2Cl + pyridine then p-CH3O · C6H4 · OH
XII
H2/Pd
XIII
HONO then KI+I2
XIV
HI
XV
I2 C2H5NH2
"Neothyroxine"
XVI

Fig. 1.

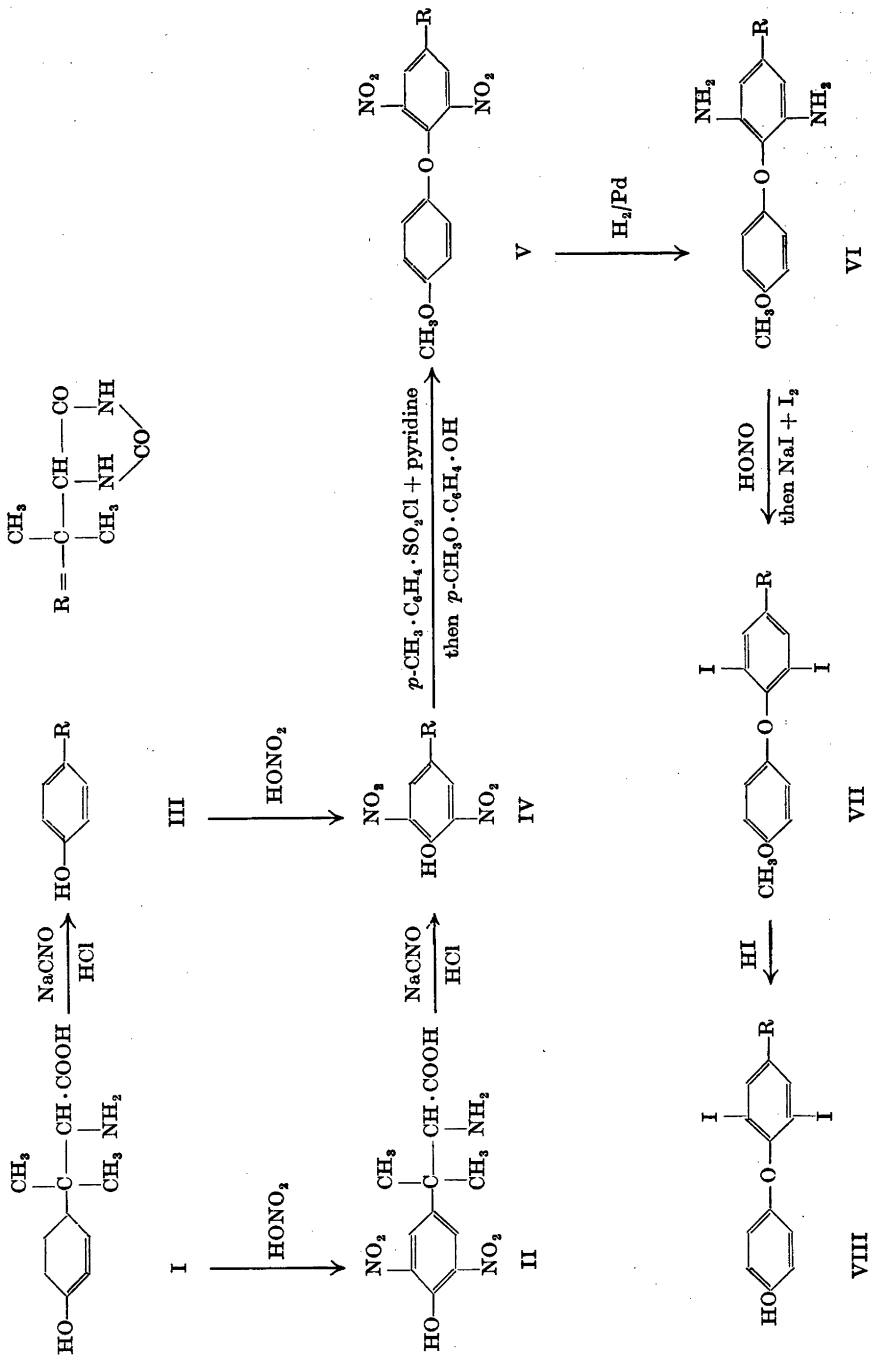

Fig. 2.

The steps involved are shown in Fig. 1. The side chain of the aminoacid was protected during these reactions by esterification of the carboxyl group and acetylation of the amino nitrogen. A series of reactions were also carried out during which the side chain was protected by conversion into a hydantoin ring. This route had, however to be abandoned as all attempts to hydrolyse 5-(α,α-dimethyl-3,5-diiodo-4-*p*-hydroxyphenoxybenzyl)hydantoin (VIII) and its methyl ether (VII) to the free aminoacid under a variety of acid and alkaline conditions failed. This was probably due to steric hindrance by the bulky substituent in position 5. (It is known that hydantoins doubly substituted in position 5 are much more resistant to hydrolysis than the monosubstituted ones. Probably a single *tertiary* alkyl group has a similar effect.) The reaction sequence is shown in Fig. 2.

The intermediate N-acetyl-3,5-dinitro-"*neo*tyrosine" (IX) was prepared by two ways, either by nitration of "*neo*tyrosine" followed by acetylation, or by carrying out these steps in the opposite order. Though it normally gave somewhat lower yield the latter method is to be preferred because the nitration of "*neo*tyrosine" was a little erratic. In most cases high yields were obtained but in a few experiments were almost zero. The reason for this could not be found.

The esterification of (X) met with some difficulties, as it was found that esterification by azeotropic distillation with chloroform, found so useful for N-acetyl-3,5-dinitrotyrosine by Chalmers *et al.*[5], was not successful with the α,α-dimethyl analogue. Esterification in a large excess of ethanol with *p*-toluenesulphonic acid as catalyst afforded a low yield of the ester; the main product was a neutral solid, which was not further investigated. (The authors cited report, that a similar result was obtained, when they tried to esterify N-acetyl-3,5-dinitrotyrosine under Fischer-Speier conditions.) It was ultimately found that esterification with a mixture of ethanol and thionyl chloride afforded the desired product in excellent yield. As the diamine (XIII) was sensitive to air at least in an impure state it was converted directly into the diiodo compound (XIV). This was obtained as a glass which could not be induced to crystallise. Simultaneous demethylation, deacetylation and hydrolysis of the ester group by hydriodic acid, however, converted the crude (XIV) into the crystalline 3,5-diiodo-D,L-"*neo*thyronine" (XV). Iodination of (XV) with iodine in aqueous ethylamine produced D,L-"neothyroxine" in a good yield.

EXPERIMENTAL *

3,5-Dinitro-D,L-"*neotyrosine*", D,L-α-*amino*-β-*(3,5-dinitro-4-hydroxyphenyl)isovaleric acid* (II). "*Neo*tyrosine" (25 g) was added in portions to concentrated sulphuric acid (90 ml) at 5 to 10°. The solution was cooled to −5° and stirred while nitric acid (17.5 ml, *d* 1.42) was added drop by drop. After the addition was complete the mixture was stirred at 0° for 15 minutes, poured onto crushed ice (400 g) and the pH brought to 4.0−4.5 by careful addition of 30 % aqueous sodium hydroxide solution, the temperature being kept below 20°. After some hours the precipitate was collected, washed with water and dried. Yellow needles. Yield 32 g (89 %). For analysis a sample was crystallised from aqueous ethanol and then melted at 222° (decomp.). (Found: C 44.2; H 4.63; N 13.8. $C_{11}H_{13}N_3O_7$ requires: C 44.2; H 4.38; N 14.0.)

* All melting points uncorrected. Petrol refers to the fraction b.p. 40—60°.

*N-Acetyl-*D,L-*"neotyrosine"* (IX). D,L-*"Neo*tyrosine (20.9 g) was dissolved in 2 *N* sodium hydroxide (225 ml) and acetylated below 20° by dropwise addition of acetic anhydride (20 ml) with constant stirring which was continued for a further hour after the addition. Excess anhydride was destroyed by stirring at 40° for ½ hour, and the reaction product was precipitated by addition of 6 *N* hydrochloric acid to the cooled solution. After storing overnight in the refrigerator the solid was filtered off. An additional amount could be obtained on concentration of the mother liquor. Total yield of product melting at 178–180° (lit.[3] 181–181.5°) 20.0 g (83 %).

*N-Acetyl-3,5-dinitro-*D,L-*"neotyrosine"* (X). *a) From N-acetyl-*D,L-*"neotyrosine"*. The foregoing N-acetylderivative (18.0 g) was added to conc. sulphuric acid (75 ml) with stirring below 10°. Nitric acid (*d* 1.42; 10 ml) was added drop by drop below −5°. Stirring was continued for one further hour after complete addition, and the reaction mixture was then poured onto ice. After some hours the solid was filtered off, carefully washed with water and dried. Yield 20.8 g (80 %). For analysis the compound was crystallised from aqueous ethanol, yellow needles, m. p. 222–225°. (Found: C 45.6; H 4.68; N 12.2. $C_{13}H_{15}N_3O_8$ requires: C 45.7; H 4.43; N 12.3.)

b) *From 3,5-dinitro-*D,L-*"neotyrosine"*. The nitrocompound was acetylated as described above for *"neo*tyrosine". Yield 90 %. M. p. 222–225° alone and in admixture with the compound just described.

*N-Acetyl-3,5-dinitro-*D,L-*"neotyrosine" ethyl ester* (XI). Thionyl chloride (7.0 ml) was added to absolute ethanol (60 ml) in dioxan (100 ml). After 15 minutes the foregoing acetylamino acid (32 g) was added and the mixture heated for 3 hours on a steam bath. Dioxan and excess ethanol were removed under reduced pressure, and the residual solid crystallised from aqueous ethanol. Yield 27.2 g (79 %) of yellow needles, m. p. 145–145.5°. (Found: N 11.2; C_2H_5O 12.0. $C_{15}H_{19}N_3O_8$ requires: N 11.4; C_2H_5O 12.2.)

*N-Acetyl-3,5-dinitro-4-p-methoxyphenoxy-*D,L-*"neophenylalanine" ethyl ester* (XII). The foregoing ester (36 g) and *p*-toluenesulphonyl chloride (19.5 g) in dry pyridine (125 ml) were heated on the water bath for ½ hour. *p*-Methoxyphenol (36 g) was added and the mixture was refluxed in an oil bath for 1 ½ hours. The solvent was removed *in vacuo* on the water bath and the residual dark oil dissolved in chloroform and washed in turn with *N* sodium hydroxide, 2 *N* hydrochloric acid and water. After drying (Na_2SO_4) the solution was filtered through a short column of aluminium oxide. Evaporation of the solvent yielded a solid, which was crystallised from aqueous ethanol and then formed yellow needles, m. p. 124–126°. Yield 33.0 g (74 %). For analysis a sample was repeatedly recrystallised and then melted at 127–128°. (Found: C 55.7; H 5.45; N 8.65. $C_{22}H_{25}N_3O_9$ requires: C 55.6; H 5.30; N 8.84.)

*3,5-Diiodo-*D,L-*"neothyronine"* (XV). The foregoing diphenyl derivative (19.5 g) in glacial acetic acid (200 ml) was hydrogenated at room temperature and pressure using a 10 % palladium on charcoal catalyst (2 g). The filtered solution was evaporated to approximately 50 ml under reduced pressure in a nitrogen atmosphere and added with stirring to conc. sulphuric acid (25 ml) below 25°. This solution was added drop by drop below 0° with constant stirring to a solution of nitrosylsulphuric acid prepared by dissolving sodium nitrite (9 g) in conc. sulphuric acid (60 ml) and dilution of this mixture with glacial acetic acid (125 ml) below 20°. Stirring was continued for a further hour after complete addition and then urea (5 g) was added. The solution was stirred for 15 minutes and then slowly poured into a stirred solution of iodine (35 g) and potassium iodide (45 g) in water (600 ml) covering a layer of chloroform (150 ml). The addition required 15 minutes, stirring being continued for one hour after complete addition. The chloroform was separated, and the aqueous layer extracted twice with chloroform. The combined chloroform solutions were washed with water, saturated with sulphur dioxide and again washed with water. The solvent was removed on the water bath, and the residual dark oil dissolved in a little benzene and filtered through a short column of aluminium oxide. The column was washed with much benzene, and the combined filtrates freed from solvent under reduced pressure. A yellow glass (15.1 g) was obtained which could not be induced to crystallise. It was refluxed for 8 hours with 57 % hydriodic acid (50 ml) and acetic acid (50 ml). The solution was evaporated *in vacuo* on the water bath and the residual syrup was dissolved in hot 50 % ethanol (75 ml) and treated with sulphur dioxide. The amino acid was precipitated from this solution by the addition of a saturated solution of sodium acetate and filtered off after keeping for some hours in the refrigerator. The crude material was purified by dissolving in boiling 50 % ethanol containing a little conc.

hydrochloric acid and decolourising with animal charcoal and then reprecipitated by the addition of a boiling solution of sodium acetate containing a little sodium sulphite to give a faintly greyish crystalline powder which melted with decomposition at 218—220°. Yield 12.2 g (53 % calculated on the nitroderivative). (Found: C 38.2; H 3.52; I 44.3. $C_{17}H_{17}I_2NO_4$ requires: C 36.9; H 3.10; I 45.9.)

D,L-"*Neothyroxine*" (XVI). The foregoing di-iodo compound (4.2 g) was dissolved in 33 % ethylamine (50 ml) and iodinated by slow addition, with stirring, of a 1.0 *N* solution of iodine in saturated aqueous potassium iodide (30.5 ml). After a short while the solution was diluted with water (200 ml) and the "*neo*thyroxine", the ethylamine salt of which had started to separate, was precipitated by addition of 4 *N* hydrochloric acid to pH 4—5. The crude product was collected after some hours and washed well with water. It was purified by dissolving in a boiling mixture of ethanol (125 ml) and 2 *N* sodium hydroxide (50 ml), decolourising with animal charcoal, filtering and reprecipitation by the addition of boiling 2 *N* hydrochloric acid to pH 4—5. Faintly yellowish crystal powder, which decomposed without melting at 192—195°. Yield 4.9 g (80 %). (Found C 25.7; H 1.82; I 61.2. $C_{17}H_{15}I_4NO_4$ requires: C 25.4; H 1.88; I 63.1.)

D,L-*5-(α,α-Dimethyl-4-hydroxybenzyl)hydantoin* (III). D,L-"*Neo*tyrosine" (31 g), sodium cyanate (20 g), and water (150 ml) were refluxed for 15 minutes, additional sodium cyanate (10 g) was added, and the mixture refluxed for a further hour, cooled and acidified with conc. hydrochloric acid. After a few hours the colourless precipitate was collected and refluxed for one hour with 6 *N* hydrochloric acid (150 ml). After cooling the solid was filtered off and washed with water. Crystallisation from aqueous acetic acid yielded small colourless needles, (24 g, 69 %), m. p. 290—295° (decomp.). (Found: C 61.6; H 6.26; N 12.0. $C_{12}H_{14}N_2O_3$ requires: C 61.5; H 6.03; N 12.0.)

D,L-*5-(α,α-Dimethyl-3,5-dinitro-4-hydroxybenzyl)hydantoin* (IV). *a) From 3,5-dinitro-*D,L-"*neotyrosine*". The hydantoin was obtained in 75 % yield when 3,5-dinitro-D,L-"*neo*tyrosine" was treated as described above for D,L-"*neo*tyrosine". Yellow prisms from ethanol, m. p. 236—237° (decomp.). (Found: C 44.7; H 3.99; N 17.3 $C_{12}H_{12}N_4O_7$ requires: C 44.5; H 3.73; N 17.3.)

b) From D,L-*5-(α,α-dimethyl-4-hydroxybenzyl)hydantoin.* This hydantoin (20 g) was added with stirring to nitric acid (100 ml, *d* 1.42) at 30—40°. After the addition the mixture was stirred for a further 2 hours and then poured into ice and water (400 ml). The solid was filtered off, washed and dried. Yield 17.5 g (63 %). M. p. and mixed m. p. with a specimen prepared by method a) 234—236°.

D,L-*5-(α,α-Dimethyl-3,5-dinitro-4-p-methoxyphenoxybenzyl)hydantoin* (V). The foregoing dinitrohydantoin (25 g), *p*-toluenesulphonyl chloride (20 g) and dry pyridine (125 ml) were mixed and refluxed on an oil bath for 15 minutes. *p*-Methoxyphenol (36 g) was added and the solution refluxed for a further hour. Most of the pyridine was removed *in vacuo* on a water bath, and the residual oil poured into *N* hydrochloric acid (500 ml). The dark oil, which separated, crystallised on cooling and scratching. The solid was filtered off, carefully washed with *N* hydrochloric acid and water, dried, and dissolved in a little boiling acetone. Some undissolved material was filtered off, and water was added to the hot solution to turbidity. The solution was allowed to cool very slowly overnight, and the yellow needles (22 g) collected. Recrystallised from aqueous acetone the product melted at 133—134°. (Found: CH_3O 7.0; N 12.8. $C_{19}H_{18}N_4O_8$ requires: CH_3O 7.2; N 13.0.)

D,L-*5-(α,α-Dimethyl-3,5-diamino-4-p-methoxyphenoxybenzyl)hydantoin* (VI). The foregoing diphenyl ether (10.4 g) in glacial acetic acid (250 ml) was hydrogenated at room temperature and pressure with 10 % palladium on charcoal catalyst (1 g). The hydrogen uptake was very slow but quantitative. After complete reduction the catalyst was filtered off and the filtrate evaporated *in vacuo*, these and subsequent operations being carried out in an atmosphere of nitrogen. A light brown gum was obtained, which was dissolved in anhydrous ethanolic hydrogen chloride, evaporated to dryness *in vacuo*, freed from excess hydrogen chloride by drying *in vacuo* overnight over potassium hydroxide, and dissolved in a small volume of anhydrous ethanol. Careful addition of much dry ether yielded a solid precipitate, which was filtered off, washed with ether, and dried *in vacuo*. Yield 9.8 g. As the product was very difficult to purify and sensitive to air when moist, it was used for the next step without further purification. (For this purpose the original gum after evaporation of the acetic acid could be used as well.)

D,L-*5-(α,α-Dimethyl-3,5-diiodo-4-p-methoxyphenoxybenzyl)hydantoin* (VII). The foregoing diamine hydrochloride (7.5 g) was dissolved in a mixture of glacial acetic acid (70 ml) and concentrated sulphuric acid (70 ml) and added dropwise with stirring below −2° to a solution of nitrosylsulphuric acid, prepared by dissolving sodium nitrite (3.5 g) in cold concentrated sulphuric acid (40 ml) and diluting with glacial acetic acid (40 ml). Stirring and cooling was continued for one hour after the addition was complete, and excess diazotising agent was destroyed by addition of a little urea and stirring for 15 minutes. This mixture was added slowly to a stirred solution of sodium iodide (20 g) and iodine (21 g) in water (350 ml), the temperature being kept below 40° by addition of ice. Stirring was continued for a further 4 to 6 hours, and the clear solution was decanted from the heavy, dark oil, which was ground with sodium bisulphite solution until completely solid and free from excess iodine. The solid was collected and thoroughly washed with water. Faintly brown crystal powder. Yield 9.2 g. This crude product was further purified by filtration in acetone solution through a short column of aluminium oxide. The filtrate was evaporated to dryness, and the solid residue crystallised from chloroform-petrol. Minute, colourless platelets, m. p. 258−259° (decomp.) (7.2 g). (Found: CH_3O 5.32; N 4.70. $C_{19}H_{18}I_2N_2O_4$ requires: CH_3O 5.24; N 4.73.)

D,L-*5-(α,α-Dimethyl-3,5-diiodo-4-p-hydroxyphenoxybenzyl)hydantoin* (VIII). The foregoing methoxydiiodocompound (7.0 g) was refluxed with a mixture of glacial acetic acid (20 ml) and 57 % hydriodic acid (20 ml). It dissolved rapidly, and the hydroxycompound soon started to separate. Heating was continued for one hour, water was added to turbidity and the mixture was stored overnight in the refrigerator. The crystals were collected and washed with aqueous acetic acid. Clusters of minute colourless spears. Yield 5.7 g. For analysis a sample was crystallised from aqueous acetic acid. The decomposition point of the compound varied between 280−290°. (Found: C 37.6; H 2.9; I 43.8; N 4.9. $C_{16}H_{16}I_2N_2O_4$ requires: C 37.4; H 2.8; I 43.9; N 4.8.)

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Studies on Antimetabolites

VII *. Note on some Reaction Products Formed in the Oxidation of 4,6-Di-(α,α-dimethylbenzyl)pyrogallol with Alkaline Potassium Permanganate

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As reported earlier the oxidation of 4,6-di-(α,α-dimethylbenzyl)-pyrogallol with alkaline potassium permanganate yielded α-oxo-β-phenyl*iso*valeric acid as the main product. In addition there were isolated some α-phenyl*iso*butyric acid and two further acid products. These have now been subjected to a more detailed study and the structures II and III are proposed.

In Part I of this series it was reported that the oxidation of 4,6-di-*tert*alkyl substituted pyrogallols with alkaline potassium permanganate in aqueous solution afforded fairly good yields of the corresponding tri-substituted pyruvic acids as well as some of the α-phenyl*iso*butyric acid derivatives. The oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol (I) was studied in some detail and in addition to the ketoacid and α-phenyl*iso*butyric acid two further acid products, here termed A and B, were isolated from the reaction mixture. It is the purpose of the present communication to describe some reactions carried out with these products, and to propose probable structures.

Compound A melts at 174—175° and has the composition $C_{23}H_{24}O_3$. It does not dissolve in aqueous sodium bicarbonate solution and thus contains no carboxyl group, but dissolves readily in *N* sodium hydroxide. With ferric chloride an intense red colouration is obtained indicating the presence of enolic hydroxyl groups. Dimethyl sulphate and alkali converts the compound into a neutral mono-methyl ether, which does not give this colour reaction. From this evidence and the close analogy of the substance with a compound obtained by Campbell by aerial oxidation of 4,6-di-*tert*butylpyrogallol in alkaline solution for which he, as a result of thorough studies [2,3], suggests structure (IV) compound A is probably an enol of 3,5-di-(α,α-dimethylbenzyl)*cyclo*pentatrione-1,2,4.

Compound B, $C_{23}H_{26}O_6$, melts at 185—186° (decomp.) and is readily soluble in sodium bicarbonate solution. It gives a mono-*cyclo*hexylammonium salt and

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a mono-piperidiniumsalt with excess of the basic reagents. With an excess of diazomethane it forms a mono-methyl derivative, $C_{23}H_{25}O_5(OCH_3)$, from which compound B is recovered after alkaline hydrolysis. Hence compound B obviously contains one free carboxyl group but no phenolic hydroxyl group. The presence of a free carboxyl is also demonstrated by titration with 0.1 *N* sodium hydroxide using phenolphthalein as indicator. One mole of alkali is consumed and the end-point of the titration is sharp. When compound B is boiled with 6 *N* sodium hydroxide, however, a solid di-sodium salt, $Na_2C_{23}H_{26}O_7$, separates. An aqueous solution of this salt has a strongly alkaline reaction, and compound B is recovered when the solution is made slightly acid, even in the cold. This behaviour indicates, that compound B, in addition to the free carboxyl group, contains a lactone group. A lactone (or ester) group is also suggested by the fact, that B gives a positive hydroxamate test.

All attempts to detect carbonyl functions in compound B by treatment with 2,4-dinitrophenylhydrazine or hydroxylamine were unsuccessful. Neither B nor its methyl ester gave any colouration with ferric chloride. Obviously they do not contain any enolisable carbonyl groups, and probably no other carbonyl groups. (Compound A, however, likewise failed to react with phenylhydrazine or hydroxylamine.)

When heated slightly above its melting point compound B readily loses one mole of water and is converted into an anhydrocompound, $C_{23}H_{24}O_5$, m.p. 187°. When dissolved in ethanol and treated with dilute sodium hydroxide this substance slowly consumes one mole of alkali and on acidification of the solution compound B is precipitated. The anhydrocompound is obviously the di-lactone corresponding to the mono-lactoneacid B. The presence of a free hydroxyl group in the anhydrocompound was shown by a Zerewitinoff determination, which indicated one "active" hydrogen atom.

Extensive oxidation of compound B with potassium permanganate in hot, strongly alkaline solution affords α-phenyl*iso*butyric acid. This proves, that the structural unit $C_6H_5—C(CH_3)_2—C$ is still present in compound B and the yield (58 % of theory) proves that there are two such groupings in the molecule.

From the evidence produced there seems to be little doubt, that compound B possesses structure III. The anhydrocompound must then be the dilactone V. The relation between compound B and the anhydrocompound V is also demonstrated by the formation from both the anhydrocompound and the methyl ester of compound B of the same reduction product, $C_{23}H_{32}O_5$, with lithium-aluminium hydride, the structure of which must be VI. The proposed structures are further supported by the infrared absorption spectrum of the anhydrocompound which in addition to the benzene bands shows strong bands at 2.82, 5.56 and 7.27 μ indicating the presence of respectively hydroxyl groups, α-lactone rings and methyl groups. There are no other bands in the 2.5 to 8 μ region.

Studies of molecular models allow certain conclusions to be drawn regarding the stereochemistry of the compounds. If in Formula III the lactone ring is considered to be oriented in the plane of the paper then the hydroxyl group on carbon atom 1 and the carboxyl on 3 must be on the same side of this plane in order to allow the ring closure to compound V. The two R-groups must both be on the opposite side to the above hydroxyl and carboxyl groups.

Nothing can be said about the orientation of the hydroxyl group attached to carbon atom 2. Theoretically two pairs of racemates may be formed when compounds V and VII are reconverted to III. The compound actually obtained, however, is homogeneous, which proves that the ring closure occurs with selective formation of one of these D,L-forms. In compounds V, VI and VII carbon atom 2 is not asymmetric, hence this uncertainty disappears, and these compounds are therefore derivatives of D,L-arabitol.

The behaviour of compound B and the pentol towards periodic acid in aqueous and dilute acetic acid solution was contradictory to the above arguments in favour of the proposed structures. The compounds were very slowly attacked and even after several days at room temperature the periodate consumption was only a fraction of that calculated. These results are surprising in view of the fact, that the diol (VIII), prepared by reduction of ethyl α-oxo-β-phenyl*iso*valerate with lithium-aluminium hydride, was rapidly and quantitatively split with the consumption of one mole of periodate. Studies on molecular models of compound B and the pentol show, however, that the bulky α-phenyl*iso*propyl groups leave very little space for the formation of an addition complex with the rather large periodate ion. The slow oxidation may thus be due to "steric hindrance". It is well-known, that the reaction velocity of the periodate oxidation of glycols is highly influenced by bulky neighbouring substituents (see *e.g.* Ref.[4]).

The low yield of the crystalline products II and III compared with the oily mixtures obtained on evaporation of the mother liquors suggests that further acid products (*e.g.* isomers of III) may have been formed in the oxidation of the pyrogallol derivative. This assumption is supported by the material balance. The yields of the various products obtained from 300 g of the pyrogallol derivative were:

Ethyl-α-oxo-β-phenyl*iso*valerate	118 g =	33 %
α-Phenyl*iso*butyric acid	24	9
Compound II	21	7
Compound III	15	4.5
		53.5 %

(The yields have been calculated as C_6H_5—$C(CH_3)_2$-groups recovered as the respective compound.)

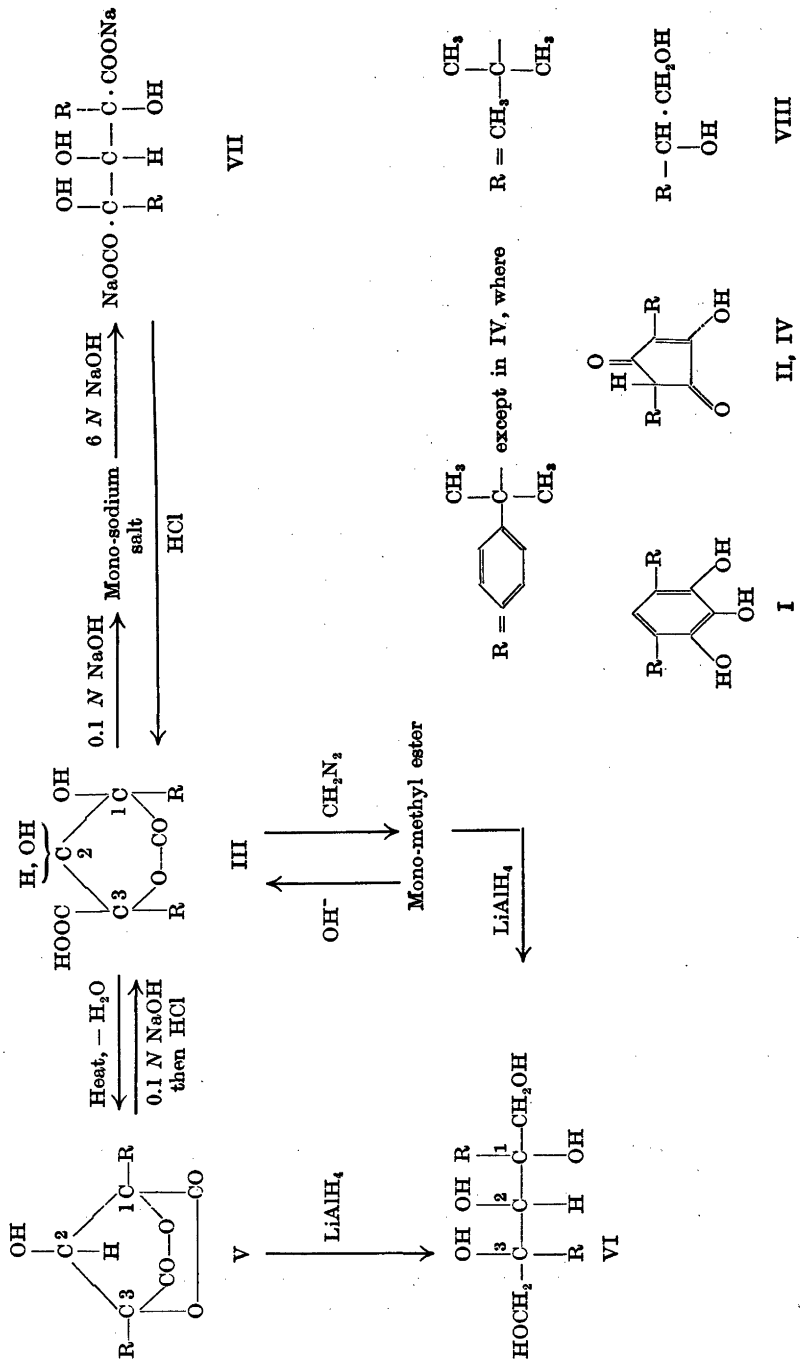
EXPERIMENTAL *

Investigation of compound A, m.p. 174—175°

Compound A was isolated as described earlier [1]. The substance was obtained as colourless needles, m. p. 174—175°, with the composition $C_{23}H_{24}O_3$. (Found: C 79.4; H 7.00; neutr. equiv. 347. $C_{23}H_{24}O_3$ requires: C 79.3; H 6.94; neutr.equiv. 348.4.) It was insoluble in sodium bicarbonate solution but dissolved readily in *N* sodium hydroxide. It gave an intense crimson colouration with ferric chloride, but did not react with hydroxylamine or 2,4-dinitrophenylhydrazine.

Cyclohexylammonium salt. On addition of excess *cyclo*hexylamine to an ethereal solution of A a mono *cyclo*hexylammonium salt separated. Needles from ethanol, m. p. 186—188° (decomp.) (Found: N 3.10. $C_{29}H_{37}NO_3$ requires: N 3.13.)

* All melting points uncorrected. Petrol refers to the fraction b.p. 40—60°.


Heat, – H2O
0.1 N NaOH then HCl
V
LiAlH4
VI
III
CH2N2
OH⁻
Mono-methyl ester
LiAlH4
0.1 N NaOH
Mono-sodium salt
6 N NaOH
HCl
VII
except in IV, where
R =
R = CH3
II, IV
I
VIII

Methyl ether. Treatment of A in methanol with an excess of dimethyl sulphate and alkali gave a high yield of a neutral monomethyl ether. Needles from petrol, m. p. 72.5—73.5°. (Found: C 80.2; H 7.28; CH_3O 8.57. $C_{24}H_{26}O_3$ requires: C 79.5; H 7.23; CH_3O 8.56.) The compound gave no colouration with ferric chloride.

Investigation of compound B, m.p. 185—186°

Compound B was isolated as described earlier [1] and obtained as colourless needles from ethanol, m. p. 185—186°, with the composition $C_{22}H_{26}O_6$. (Found: C 69.2, 69.0; H 6.20, 6.55; neutr.equiv. 397/phenolphthalein. $C_{22}H_{26}O_6$ requires: C 69.3; H 6.58; neutr. equiv. 398.4.) The compound was insoluble in water, sparingly soluble in hot benzene and readily so in hot ethanol. It gave a positive hydroxamate test for ester groups and a yellow precipitate with ferric chloride.

Cyclohexylammonium salt. When an excess *cyclo*hexylamine was added to an alcoholic solution of B a mono-*cyclo*hexylammonium salt separated as long, thin needles. M. p. 213—215° (decomp.). (Found: C 69.8; H 8.21; N 2.74. $C_{28}H_{39}NO_6$ requires: C 70.0; H 8.20; N 2.82.)

Piperidinium salt. This salt was similarly prepared from B and piperidine in ethanol. Needles, which melted at 141—143°, resolidified at about 170° and then melted at 225—228°, or rhombic plates, m. p. 225—228°. (Found: C 69.4; H 7.67; N 2.85. $C_{26}H_{37}NO_6$ requires: C 69.5; H 7.71; N 2.90.)

Disodium salt. When B (1.0 g) was dissolved in the minimum amount of hot *N* sodium hydroxide, and the solution was added to boiling 6 *N* sodium hydroxide (25 ml) a disodium salt soon started to separate. After cooling the salt was filtered off, rapidly washed with ice-cold water, acetone and ether and dried. (Found: C 58.6; H 6.1; Na 10.2; neutr. equiv. 462. $C_{25}H_{26}O_7Na_2$ requires: C 60.0; H 5.7; Na 10.0; neutr. equiv. 460.4.) The neutralisation equivalent of the salt was determined by dissolving the compound in aqueous ethanol, adding standard 0.1 *N* hydrochloric acid and titrating the excess acid with 0.1 *N* sodium hydroxide, using phenolphthalein as indicator. — When an aqueous solution of this salt was acidified with hydrochloric acid compound B separated (identified by mixed m. p.).

Methyl ester of B. When an ethereal solution of III was left overnight with an excess diazomethane a monomethyl ester formed. Needles from benzene-petrol. M. p. 162°. (Found: C 69.9; H 7.10; CH_3O 7.6. $C_{24}H_{28}O_6$ requires: C 69.9; H 6.84; CH_3O 7.5.) The ester gave no colouration with ferric chloride. When refluxed with 2 *N* aqueous-ethanolic sodium hydroxide it was hydrolysed, and the acid B separated on acidification of the solution.

Dehydration of compound B. Compound B (3.0 g) was heated in a test tube inserted in an oil bath kept at 190—200° until the evolution of vapour ceased. The reaction product was crystallised from benzene-petrol. Needles (2.3 g), m. p. 187—187.5°. (Found: C 73.0; H 6.28; "active H" 0.8. $C_{22}H_{24}O_5$ requires: C 72.6; H 6.30.) In 0.1 *N* aqueous sodium hydroxide the compound slowly consumed alkali and dissolved. (Found: neutr. equiv. 384. Calculated: 380.4.)

Reduction of the methyl ester of B with lithium-aluminium hydride. The ester (3.0 g) in absolute ether (75 ml) was added with stirring to a suspension of lithium-aluminium hydride (2 g) in absolute ether (50 ml) and the mixture was refluxed overnight on the water bath. Excess hydride was destroyed by careful addition of water and the precipitated salts were dissolved with 4 *N* hydrochloric acid. The ether layer, containing the suspended reaction product, was separated from the aqueous solution, and the solid filtered off and crystallised from aqueous ethanol. Colourless needles (2.1 g) m. p. 163—164°. (Found: C 71.6; H 8.01. $C_{22}H_{32}O_5$ requires: C 71.1; H 8.30.) The compound was almost insoluble in ether and water, readily soluble in ethanol. Gentle heating of the compound with concentrated sulphuric acid produced an intense red colouration. Attempts to acetylate the compound invariably led to mixtures.

Reduction of the dehydration product from B with lithium-aluminium hydride. When the dehydration product from B was treated as just described for the ester of B a polyol resulted, m. p. 163°, which was found by mixed melting point determination to be identical with the reduction product from this ester.

Oxidation of B with alkaline potassium permanganate. Compound B (2.5 g) was refluxed with a solution of potassium hydroxide (15 g) in water (50 ml) until a solid salt started to separate. Powdered potassium permanganate (25 g) was added and the mixture heated overnight on the water bath. Excess permanganate was destroyed with sulphur dioxide, the solution acidified with dilute sulphuric acid, and repeatedly extracted with ether. On evaporation of the ether a solid was obtained, which was crystallised once from petrol. Yield 1.2 g. Several crystallisations gave needles, m. p. 78° undepressed by *α*-phenyl*iso*-butyric acid.

D,L-*1,2-dihydroxy-3-methyl-3-phenylbutane* (VIII). Ethyl *α*-oxo-*β*-phenyl*iso*valerate (20 g) in absolute ether (50 ml) was added with stirring to a suspension of lithium-aluminium hydride (7.0 g) in absolute ether (100 ml). The mixture was refluxed overnight, excess hydride was destroyed by careful addition of water, and the precipitated salts were dissolved by addition of 6 *N* hydrochloric acid. The diol was extracted with ether, washed with water and sodium bicarbonate solution and the extract dried (K_2CO_3). Removal of the ether yielded an oil, which crystallised when cooled and scratched. Crystallisation from petrol afforded colourless needles (15.4 g), m. p. 50—51°. (Found: C 73.4; H 8.78. $C_{11}H_{16}O_2$ requires: C 73.3; H 8.95.) The compound consumed 1.0 mole of periodate when treated with periodic acid in dilute acetic acid.

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Kinetics of Complex Formation

III *. The Rate of Decomposition of Hexammine Chromium(III) Ion

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The decomposition of hexammine chromium(III) ion in nitric acid solutions is followed by measuring the rate of dissolution of the hexammine nitrate. The aquation of the hexammine is independent of the hydrogen ion concentration and the following expression is found for the pseudo-unimolecular velocity constant

$$k_{-6} = 10^{12.3} \cdot e^{-26\,000/RT}$$

By spectrophotometric analysis it is proved that the hexammine decomposes by going through a series of lower ammines.

The complexity of the chromium(III)-ammines can apparently not be determined in aqueous ammonia-ammonium buffers. The ammines undergo various transformations and under certain conditions polynuclear salts may be formed [3]. Even in solutions with very high ammonium salt concentration a slow irreversible decomposition continues which sooner or later results in the separation of some basic precipitates. It may be mentioned that a hexammine chromium(III) solution of the composition: $C_{Cr} = 0.006\ M$, $C_{NH_3} = 2\ M$, $C_{NH_4NO_3} = 5\ M$, which was kept in the dark at 68°, remained completely homogeneous for two days, but on the third day nearly all the chromium had separated as a basic precipitate. Fig. 1a shows how the absorption spectrum of this solution changes with time at 25° and 68° C. An experiment in which charcoal was added to the same solution (see Fig. 1a) shows that heterogeneous catalysis, contrary to what is found [4] for corresponding transformations of cobalt(III)-ammines, only plays a minor role in the decomposition reactions of chromium (III)-ammines. Day-light catalyzes the decomposition to some extent, and for this reason all solutions were kept in dark bottles.

Fig. 1b shows how the absorption spectra of acidic hexammine chromium(III) nitrate solutions change with time at 50°. The family of curves shows directly that the transformations proceed independent of the hydrogen

* For the earlier papers in this series, see Refs. [1,2].

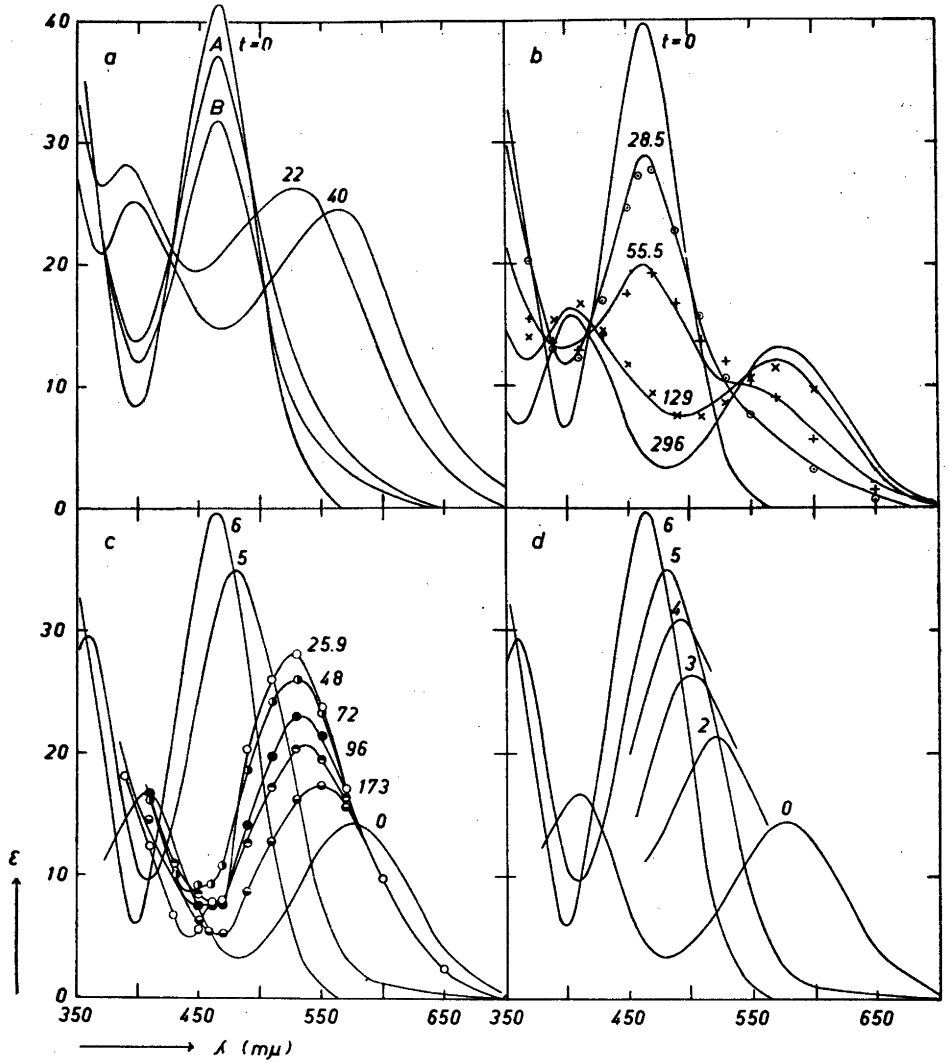

Fig. 1. Molar extinction coefficients versus wavelength in mμ.

a: A solution of the composition: $C_{Cr} = 0.006$ M, $C_{NH_3} = 2$ M, $C_{NH_4NO_3} = 5$ M *followed in time. Curve A: 100 hrs at 25° C. Curve B: 100 hrs at 25°, but with 2 g "Norite" per 100 ml. Curves after 22 and 40 hrs at 68.5° are also given.*

b: Acidic 0.005 M *hexammine chromium (III) nitrate solutions followed in time at 50° C. In 1* M HNO_3 *after 0, 28.5, 55.5, 129 and 296 hrs (full-drawn curves). In 0.5* M HNO_3 *after 29 hrs (o-points), 54.5 hrs (+ points), and in 2* M HNO_3 *after 127.5 hrs (×-points).*

c: Absorption spectra of reaction products. ε_R, λ*-curves at 40° corresponding to the experiment given in Table 1. For comparison is also given the absorption of hexammine, aquopentammine and hexaquo chromium (III) ions (curves 6, 5 and 0, respectively).*

d: Absorption spectra of aquoammine chromium (III) ions at room temperature. Hexammine and hexaquo in 1 M HNO_3 *(curves 6 and 0). Aquopentammine in 0.1* M $HClO_4$ *(curve 5) according to measurements of C. E. Schäffer. Diaquotetrammine, triaquotriammine and tetraquodiammine in water (curves 4, 3 and 2) according to Colmar and Schwartz* [5].

Table 1. Velocity of decomposition of hexammine chromium(III) ion in 1 M HNO_3 at 40° C.

t_{hours}	0	25.92	48.0	72.25	95.75	172.6
C_{NH_3}	0.1763	0.1976	0.2152	0.2359	0.2618	0.3311
$C_{NH_3}-C^{\circ}_{NH_3}$	0	0.0213	0.0389	0.0596	0.0855	0.1548
$10^8 \cdot \frac{d\ C_{Cr}}{d\ t_{sec.}}$	—	3.81	3.75	3.82	4.13	4.15
$10^6 \cdot k_{-6}$ (sec.$^{-1}$)	—	1.30	1.28	1.30	1.40	1.41

$k_{-6}(av.) = 1.34 \cdot 10^{-6}$

ion concentration in 0.5, 1 and 2 *M* nitric acid solution. After about 300 hours the absorption curve has reached an appearance which is very similar to that of the hexaquo chromium(III) ion (see Fig. 1d, where also absorption curves [5] for the intermediate chromium(III) ammines are given). The extinction coefficients in the maxima are somewhat higher than for the hexaquo ion, but polynuclear latently basic compounds perhaps exist in the solution [6].

The velocity of decomposition of the hexammine chromium(III) ion was determined directly by measuring the rate of dissolution of luteo nitrate in nitric acid solutions. Solvent and salt were vigorously shaken in a thermostat, and samples were taken from time to time, and the increase in concentration as well as the optical density at various wavelengths were determined. For convenience, the total ammonia concentration $C_{NH_3} = 6C_{Cr}$ instead of the chromium was determined by a micro-Kjeldahl distillation. Denoting the molar saturation concentrations for luteo salt with C°_{Cr} and $C^{\circ}_{NH_3}$, respectively, we have:

$$\frac{d\ C_{Cr}}{d\ time} = \frac{C_{NH_3}-C^{\circ}_{NH_3}}{6 \cdot time} = k_{-6} \cdot \frac{C^{\circ}_{NH_3}}{6}$$

and for the velocity constant

$$k_{-6} = \frac{C_{NH_3}-C^{\circ}_{NH_3}}{C^{\circ}_{NH_3} \cdot time}$$

Saturation is reached in a few minutes, and the data given in Table 1 show that it is possible by this method to get fairly constant values for the rate constant.

Denoting the optical density in general by D, and the optical density of pure hexammine in saturated solution by D°_6, we get for the mean extinction coefficient of the reaction products formed by the decomposition:

$$\varepsilon_R = \frac{D-D^{\circ}_6}{(C_{Cr}-C^{\circ}_{Cr}) \cdot d} = \frac{6\ (D-D^{\circ}_6)}{(C_{NH_3}-C^{\circ}_{NH_3}) \cdot d}$$

where d is the thickness of the absorbing layer in cm. The mean extinction coefficients of reaction products found at various times in the experiment given in Table 1 are plotted in Fig. 1c. The family of curves is not far from having an isopiestic point in common with all the known aquo ammine

Table 2. Average values for the aquation velocity constant.

$$\log k_{-6} = 12.3 - \frac{26\,000}{4.57\ T}$$

°C	$1/T°$	C°_{Cr} (solubility in 1 *M* HNO_3)	log k_{-6} ($sec.^{-1}$) obs.	calc.
25.0	0.003356	0.00964	−6.69	−6.8
40.0	0.003195	0.02938	−5.87	−5.85
63.8	0.002969	0.1260	−4.51	−4.6

chromium(III) ions except the pentammine ion (see Fig. 1d). It is, therefore, far from certain that aquo pentammine is ever important among reaction products. It is qualitatively known from direct observations that this ion is decomposed to lower ammines at a much higher rate than the luteo ion. On the other hand the relatively strong displacement of the maximum of the ε_R-curves towards red seems to exclude the possibility that the simple ammines are the only reaction products.

The mean values for k_{-6} determined at 25°, 40° and 63.8° C are shown in Table 2. By graphic interpretation of the data the activation energy E is estimated to 26 kcal/mole, and the frequency factor $Z'_{-6} = Z_{-6} \cdot C_{aq}$ to $10^{12.3}$. The values for the velocity constant computed from these values for the Arrhenii constants are also given in the table.

EXPERIMENTAL

Preparation of salts. With minor modifications the complex salts used were prepared according to methods described in the literature. Hexammine chromium(III) nitrate was prepared from the luteo chloride, which was prepared by the Christensen method from anhydrous chromium(III) chloride and liquid ammonia [7]. The hexammine nitrate was purified by repeated reprecipitations with nitric acid; the product was washed with alcohol and air-dried at 60° C, and prepared in this way it could be kept unchanged for months in a dark bottle with a tight fitting stopper. *Anal.* Calc. for $Cr(NH_3)_6(NO_6)_3$: Cr 15.3; N 37.1. Found: Cr 15.1; N 37.1. The solubility of the salt at 18.6° C was found to be 0.0117 *M* in 0.5 *M* HNO_3, 0.00543 *M* in 1.0 *M* HNO_3, and 0.00314 *M* in 2.0 *M* HNO_3.

Technique. The measurements of the rate of dissolution of the hexammine nitrate were made in a water thermostat with an arrangement for effective horizontal shaking of a bottle. At various times during an experiment the shaking was stopped for a short time, and a measured amount of the saturated solution drawn up through a glass filter, adequately diluted and cooled. In order to avoid precipitation during the transference of the saturated solution the pipette was surrounded by a glass mantle containing water at the thermostat temperature.

The optical densities were measured at a Beckman DU. Cells of 1, 2 and 5 cm were used according to wavelength and dilution.

The total ammonia concentrations of the solutions were determined by a micro-Kjeldahl distillation in a Parnas-Wagner apparatus. The solution was transferred with a syringe pipette, and the ammonia was steam-distilled into an excess of 0.01 *N* HCl. The back titration was made with a 0.01 *N* borax solution, and methylred was used as indicator. A blank was run to correct for traces of ammonia in the Kjeldahl lye.

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Polarographic Processes Involving Chemical Reaction of the Product of the Electron Transfer Reaction

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Using steady state and thermodynamical methods current-voltage equations have been derived for polarographic processes in which the product of the electron transfer reaction is involved in a subsequent chemical reaction.

The application of kinetics in the field of polarography has in recent years become increasingly popular. A better understanding of the conditions at the surface of the electrode, where the electron exchange reactions occur, has made it possible to attack problems concerning mechanisms of electrode reactions. For instance, the so-called irreversible polarographic processes, in which the electron transfer reaction is the slow step competing with the rate of diffusion, have been now thoroughly explained. Similarly, many catalytic processes that can occur at the surface of the microelectrode have been also clarified using kinetic considerations.

This investigation deals with a polarographic problem in which kinetics has been used to gain further insight into the phenomena in question. It is concerned with polarographic processes in which the product of the electron transfer reaction is involved in a subsequent chemical reaction [1]. This type of process can be represented by the general scheme:

$$\begin{gathered} A \overset{ne^-}{\rightleftharpoons} \sum B \\ \sum B + \sum C \rightleftharpoons \sum D \end{gathered} \qquad (1)$$

where A is the species undergoing reduction or oxidation and the various products of the electron transfer reaction are denoted by B, possible chemical reactants by C and the products of the chemical reaction by D. This investigation is restricted to cases where the products of the chemical reaction are not electrochemically active at the potential where A is reduced or oxidized. The so-called catalytic waves will thus be excluded from this study.

Comparatively few reports are found in the literature concerning polarographic processes involving a subsequent chemical reaction. Particulary this

is true for reduction waves, as pointed out by Tanford and Wawzonek [2]. This type of polarographic wave was first reported by Smith, Kolthoff, Wawzonek and Ruoff [3]. They studied the oxidation of hydroxychromans and hydroxycoumarans at the dropping mercury electrode. Chemical or electrochemical oxidation of these compounds produces quinones. The quinones are reversibly reduced to the corresponding hydroquinones and not to the original hydroxychromans or coumarans. On the basis of an analysis of the oxidation waves, they concluded that the electrochemical oxidation involves a reversible step, but that the oxidation product formed is unstable and is irreversibly transformed to the corresponding quinone. The authors postulated that the potential is determined by the reversible reaction

$$A + H_2O \rightleftharpoons B + 2\ H^+ + 2\ e^- \qquad (2)$$

whereas the irreversible step, the transformation of compound B into C

$$B \xrightarrow{a} C \qquad (3)$$

was pictured as an unimolecular reaction. In deriving the expression for the polarographic wave Smith *et al.* use a rather odd therminology in first considering α as a sort of equilibrium constant, $C_B = \alpha\ C_C$, but later calling it a rate constant, although it is given as a dimensionless quantity. Their equation has the form:

$$E_{d.e.} = \text{Const.} + \frac{RT}{2F} \ln \left[\frac{i}{i_d - i} \cdot \frac{k_A}{k'} \alpha \right] + \frac{RT}{2F} \ln [H^+] \qquad (4)$$

where k_A is the Ilkovic constant for compound A and k' is that for compounds B and C (diffusion coefficients practically equal).

Vavrin [4] studied a similar case in the polarographic oxidation of L-ascorbic acid. He derived an equation describing the current-voltage relationship assuming that the chemical decomposition of the oxidized form is a reversible monomolecular reaction:

$$Ox_{rev} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ox_{irr} \qquad (5)$$

where Ox_{rev} represents the form which can be reversibly reduced and Ox_{irr} that which cannot. The rate constant of the forward reaction is denoted by k_1, that of the reverse reaction by k_2. The derivation was based upon the Nernst relationship. In the customary way $(i_d - i) = k$ Red, where k is the Ilkovic constant for the reduced form. In trying to relate the current to the surface concentration of Ox_{rev}, Vavrin seems to have used an equation of doubtful validity, *viz.*,

$$i = k\ Ox_{rev} + k_1\ Ox_{rev} - k_2\ Ox_{irr} \qquad (6)$$

where k again represents the Ilkovic constant, the same for both the oxidized and reduced forms, and Ox_{rev} and Ox_{irr} are the surface concentrations of the species involved. (The two last terms in the right hand side of equation (6) have the dimension of a rate: moles/liter/sec., whereas the term $k\ Ox_{rev}$ gives amperes). Solving for Red and Ox_{rev} and inserting these quantities into the Nernst relation gives

$$E_{\text{d.e.}} = E^\circ + \frac{RT}{nF} \ln \left[\frac{i_d - i}{i} \cdot \frac{k + k_1 + k_2}{k_1 + k_2} \right] \quad (7)$$

as Vavrin's result.

Oldham [5] has studied the reduction of certain organic mercury compounds and used steady state kinetics in elucidating some of the processes involved. He found that the second wave was irreversible at low pH values but changed to a reversible one when the hydrogen ion concentration was decreased. He offered the explanation that the rate by which the reduction product reacts with hydrogen ions was at high pH values slow enough not to interfere with the reversible electron transfer reaction but competed favorably at low pH values with the back oxidation rendering the wave irreversible.

Kern [6] has derived an equation for a process in which the reduction product is transformed irreversibly to a non-electroactive form. Koutecky's [7] mathematical treatment of linear electrode reactions coupled with chemical reaction appeared in 1951.

Steady state and thermodynamical methods will be used in the following derivations. As was mentioned earlier steady state kinetics has been employed in polarography by some investigators [5,8]. At first sight it seems to be a rather crude assumption that a steady state is attained at the dropping mercury electrode with its varying surface area and diffusion layer thickness. However, relating the mean current to the mean area and concentration during the drop-life, no serious errors are introduced. The faster the electrode reactions are, the closer does the steady state method describe the real conditions at the surface of the electrode. With slower electrode processes the error becomes greater because the concentrations are not independent of time. For instance, in the case of the completely irreversible electrode reaction (no back-reaction), the steady state method does not yield the correct current-voltage relationship, as obtained by rigorous mathematical procedure. A comparison of the two methods for this case has been made by Hush and Oldham [9]. On the other hand, the steady state method gives an illustrative picture of the relative magnitudes of the rates of reactions in schemes which would be too complex to be dealt with by rigorous mathematical methods.

The thermodynamical method has to be relied upon in even more complicated cases.

THE ELECTRODE REACTION COUPLED WITH AN UNIMOLECULAR* CHEMICAL REACTION

The simplest reaction to be considered here can be represented by the following scheme:

$$\mathrm{A} \overset{k_A}{\rightleftharpoons} \mathrm{A}^\circ + ne^- \overset{k_1}{\rightleftharpoons} \mathrm{B}^\circ \overset{k_3}{\rightleftharpoons} \mathrm{C}^\circ \overset{k_c}{\rightleftharpoons} \mathrm{C} \quad (8)$$

$$\mathrm{B}^\circ \underset{k_4}{\overset{k_2}{\rightleftharpoons}} \mathrm{B}, \quad \mathrm{B} \xrightarrow{k_B}$$

* The molecularity and the order of the reaction are considered to be identical here.

where the superscript ° refers to concentrations at the electrode surface. The various processes occurring at the electrode are considered as unimolecular rate processes. The rate constants are defined as the number of moles of substance reacting in unit time, per unit area, per unit concentration, the dimension being cm sec.$^{-1}$. The heterogeneous rate constants may be related to conventional three-dimensional rate constants by considering the "thickness" of the reaction zone. Expressions for this have been derived by various authors [8,10]. In scheme (8) the constants for the electron transfer reaction are denoted by k_1 (forward) and k_2 (backward), those for the chemical reactions by k_3 (forward) and k_4 (backward). The concentrations of the species involved are designated by A, B and C. The kinetic factors [11] which involve the activity coefficients, are here considered equal to unity for the sake of simplicity. The diffusion rate constants of the species are denoted by k_A, k_B and k_C, respectively. They are defined in the following manner:

The Ilkovic equation gives the mean diffusion current

$$i_d = \frac{6}{7}\sqrt{\frac{7}{3}} \cdot \frac{1}{\sqrt{\pi}} \left(\frac{36\pi}{d^2}\right)^{1/6} nFCD^{1/2}\, m^{2/3}\, t^{1/6} \tag{9}$$

in which the notations are the conventional ones. The expression of the mean diffusion current in the steady state treatment is given by (see below)

$$i_d = n\ F\ q\ C\ k_d \tag{10}$$

where q is the mean area of the mercury drop and k_d represents the diffusion rate constant. From equations (9) and (10) one obtains

$$k_d = \sqrt{\frac{7}{3}} \cdot \frac{1}{\sqrt{\pi}} \cdot \frac{5}{3} \cdot \frac{6}{7} \sqrt{\frac{D}{t}} = 1.231 \sqrt{\frac{D}{t}} \tag{11}$$

which is the expression for the diffusion rate constants.

The potential dependence of the rate constants of the electron transfer reaction is given by

$$k_1 = k^\circ_1 \exp \frac{-\alpha nFE}{RT} \tag{12}$$

and

$$k_2 = k^\circ_2 \exp \frac{(1-\alpha) nFE}{RT} \tag{13}$$

where k°_1 and k°_2 represent the values of the rate constants at zero applied potential [12]. The transfer coefficient α is the fraction of the potential difference which effects the reduction, whereas $(1-\alpha)$ is the fraction which effects the oxidation [2,12]. The sign of the exponent is to be taken negative for reductions (increasing k for more negative or less positive potential) and positive for oxidations.

The steady state treatment requires that the concentrations of the species involved, both in the bulk of the solution and at the electrode surface, are independent of time, *i. e.*, the sum of moles brought to the electrode by diffu-

sion or reaction equals the sum of moles removed. Applying this condition to the above-mentioned scheme, the following equations are obtained:

$$Ak_A + B^\circ k_2 = A^\circ k_A + A^\circ k_1 \quad (14)$$

$$Bk_B + A^\circ k_1 + C^\circ k_4 = B^\circ (k_2 + k_3 + k_B) \quad (15)$$

$$Ck_C + B^\circ k_3 = C^\circ k_C + C^\circ k_4 \quad (16)$$

The mean cathodic current, i, is due to the difference in the rates of reduction and oxidation:

$$i = nFq\ (A^\circ k_1 - B^\circ k_2) \quad (17)$$

Using simple algebra the expression for the current can be derived from equations (14)—(17):

$$i = nFq \left[\frac{Ak_A k_1 (k_3 k_C + k_4 k_B + k_B k_C) - Bk_A k_B k_2 (k_4 + k_C) - Ck_A k_C k_2 k_4}{k_1 (k_3 k_C + k_4 k_B + k_B k_C) + k_A (k_2 k_4 + k_2 k_C + k_3 k_C + k_4 k_B + k_B k_C)} \right] \quad (18)$$

Considering potentials at which k_1 becomes very large, all the other terms except those containing k_1 are unimportant and equation (18) then takes the form

$$i_d = nFqAk_A \quad (19)$$

where i_d represents the diffusion limited mean current, *i. e.*, complete concentration polarization prevails. From equations (18) and (19) it is then possible to get

$$\frac{i_d - i}{i} = \frac{Ak_A (k_2 k_4 + k_2 k_C + k_3 k_C + k_4 k_B + k_B k_C) + Bk_B k_2 (k_4 + k_C) + Ck_C k_2 k_4}{Ak_1 (k_3 k_C + k_4 k_B + k_B k_C) - Bk_B k_2 (k_4 + k_C) - Ck_C k_2 k_4} \quad (20)$$

which reduces to

$$\frac{i_d - i}{i} = \frac{k_A (k_2 k_4 + k_2 k_C + k_3 k_C + k_4 k_B + k_B k_C)}{k_1 (k_3 k_C + k_4 k_B + k_B k_C)} \quad (21)$$

provided B and C, the concentrations of the products in the bulk of the solution, are equal to zero, which is usually the case.

Equation (21) has been derived without recourse to considerations of the relative magnitudes of the various velocity constants. It will, therefore, apply whether or not the reactions are reversible. It represents thus a general relationship from which current-voltage equations can be derived for polarographic processes coupled with a monomolecular chemical reaction.

Depending on relative magnitude of the various rate constants different situations are encountered.

If a case, where

$$k_1,\ k_2 \gg k_3,\ k_4,\ k_A,\ k_B,\ k_C \quad (22)$$

is at hand, equation (21) then takes the form

$$\frac{i_d - i}{i} = \frac{k_2}{k_1} \left[\frac{k_4 k_A + k_A k_C}{k_3 k_C + k_4 k_B + k_B k_C} \right] \quad (23)$$

since the terms in the nominator which do not contain k_3 can be neglected. Condition (22) actually represents the criterion of polarographic reversibility which requires that the rates of the electron transfer reactions are faster than the other processes involved [5, 13]. Accordingly, the current-voltage equation of this case should yield a "reversible" log plot slope.

If desired the activity coefficients can be introduced into equation (23) at this stage conveniently. They are associated with the reaction rate constants in scheme (8) as follows:

A.....f_1k_1
B.....f_2k_2, f_2k_3
C.....f_3k_4

The connection between the diffusion rate constants and the activity coefficients is of a different nature. The diffusion rate constants are derived on the basis of the Ilkovic equation which in turn is based upon Fick's laws which are valid only under ideal conditions. Therefore the change in the rates of diffusion with change in overall ionic strength must be referred to the variation of the diffusion coefficient D [14, 15]. Because of the lack of knowledge of values of D in various media, the usual practice is to consider it constant and equal to the value at infinite dilution. After inserting the activity coefficients into equation (23) it takes the form:

$$\frac{i_d - i}{i} = \frac{k_2 f_2}{k_1 f_1}\left[\frac{f_3 k_4 k_A + k_A k_C}{f_2 k_3 k_C + f_3 k_4 k_B + k_B k_C}\right] \tag{24}$$

Combining equations (12), (13) and (24) and taking logarithms, the following relation is obtained:

$$E = \frac{RT}{nF}\left[\ln \frac{k^\circ_1}{k^\circ_2} + \ln \frac{f_1}{f_2} + \ln \frac{f_2 k_3 k_C + f_3 k_4 k_B + k_B k_C}{f_3 k_4 k_A + k_A k_C}\right] + \frac{RT}{nF} \ln \frac{i_d - i}{i} \tag{25}$$

The three terms in the parenthesis of the right hand side of equation (25) are constant for a given solution and reference electrode and they represent the half-wave potential of the reaction. Thus

$$E = E_{\frac{1}{2}} + \frac{RT}{nF} \ln \frac{i_d - i}{i} \tag{26}$$

which is the familiar equation for a reversible polarographic wave.

It has been shown that a first-order reversible chemical reaction which follows a reversible electron transfer reaction, does not alter the shape of the polarographic wave provided k_1 and k_2 are much larger than the other rate constants. The half-wave potential, however, is a function of the magnitude of the rate constants of the chemical reaction and of the diffusion rate constants.

When the first-order chemical reaction is very rapid fulfilling the condition

$$k_1,\ k_2, \gg\ll k_3,\ k_4, \gg k_A,\ k_B,\ k_C \tag{27}$$

equation (21) then takes the form

$$\frac{i_d - i}{i} = \frac{k_2}{k_1} \cdot \frac{k_4 k_A}{k_3 k_C + k_4 k_B} \tag{28}$$

from which one obtains

$$E_{\frac{1}{2}} = \frac{RT}{nF}\left[\ln \frac{k^\circ_1}{k^\circ_2} + \ln \frac{f_1}{f_2} + \ln \frac{f_2 k_3 k_C + f_3 k_4 k_B}{f_3 k_4 k_A}\right] \tag{29}$$

That the same result is found whether the rate of the chemical reaction is faster or slower than the electron transfer reaction actually means that the system is in complete equilibrium. Again the wave is a reversible one. This is to be credited to the properties of a first-order reaction; under similar conditions, second-order reactions do not in general give reversible waves.

Thermodynamic consideration of case (27) results in an identical conclusion:

The Nernst relationship which presumes reversibility of the electrode reaction, is given by

$$E = E' + \frac{RT}{nF} \ln \frac{A^\circ}{B^\circ} \tag{30}$$

where E' denotes the standard potential of reaction

$$A + ne^- \rightleftharpoons B \tag{31}$$

and the superscript ° refers to surface concentrations. According to usual polarographic practice one has that $A^\circ = (i_d - i)/k'_A$. On the other hand, the current is proportional to the sum of the concentrations of the two forms of the reduction product, provided the diffusion coefficients are nearly equal:

$$i = k'_{B,C}\ (B^\circ + C^\circ) \tag{32}$$

Using homogeneous rate constants, one may write for the chemical reaction

$$\frac{C}{B} = \frac{k_3}{k_4} = K \tag{33}$$

Hence

$$B^\circ = \frac{i}{k'_{B,C}\ (1 + K)} \tag{34}$$

and inserting the expressions for A° and B° into equation (30) one has for the half-wave potential

$$E_{\frac{1}{2}} = E' + \frac{RT}{nF} \ln \frac{k'_{B,C}\ (k_3 + k_4)}{k'_A \cdot k_4} \tag{35}$$

which is identical with equation (29) save for the activity coefficients. The case studied by Vavrin [4] falls under the category represented by condition (27). It is the opinion of the author that Vavrin should have used equation (32) rather than (6) to get the correct result.

When the first-order chemical reaction is irreversible the case can be that the following condition is fulfilled

$$k_1,\ k_2,\ \gg k_3,\ \gg k_A,\ k_B,\ k_C \tag{36}$$

From equation (21) it is then readily seen that

$$\frac{i_d - i}{i} = \frac{k_2}{k_1} \cdot \frac{k_A}{k_3} \tag{37}$$

and the expression for the half-wave potential becomes

$$E_{\frac{1}{2}} = \frac{RT}{nF}\left[\ln \frac{k^\circ_1}{k^\circ_2} + \ln \frac{f_1}{f_2} + \ln \frac{k_3}{k_A}\right] \tag{38}$$

Here the wave is reversible and the half-wave potential is a function of k_3. Similarly, a reversible wave is obtained when

$$k_1,\ k_2 \gg k_3 \simeq k_A,\ k_B,\ k_C \tag{39}$$

viz.,

$$E_{\frac{1}{2}} = \frac{RT}{nF}\left[\ln \frac{k^\circ_1}{k^\circ_2} + \ln \frac{f_1}{f_2} + \ln \frac{k_3 + k_B}{k_A}\right] \tag{40}$$

If, on the other hand, k_3 is of the same order of magnitude or greater than k_2, then the wave is irreversible, *e. g.*,

$$\frac{i_d - i}{i} = \frac{k_A}{k_1} \tag{41}$$

and thus

$$E = \frac{RT}{\alpha nF}\left[\ln k^\circ_1 + \ln \frac{f_1}{k_A}\right] + \frac{RT}{\alpha nF} \ln \frac{i_d - i}{i} \tag{42}$$

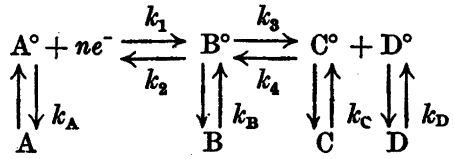
which is the equation for an irreversible polarographic wave with an "irreversible" log plot slope of $0.0591 / \alpha n$ volt.

The case investigated by Smith *et al.*[3] is one obeying condition (36). It is clear that when the rate of transformation is much faster than diffusion, k_3 will dominate and the half-wave potential will a function of it and not of the rate of diffusion. Considering $i = \alpha C_B$ in the case of Smith *et al.* one arrives at the result given by equation (38).

Equation (38) is in agreement with the equation presented by Kern[6] provided k_3 is replaced by μk_{hom}. The thickness of the reaction zone, μ, is equal to $\sqrt{D_B / k_{hom}}$ and k_{hom} is the homogeneous rate constant of the chemical decomposition. Note that Kern's case is an oxidation.

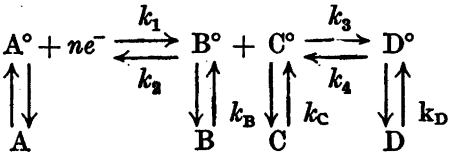
THE ELECTRODE REACTION COUPLED WITH A BIMOLECULAR CHEMICAL REACTION

Three different types of reaction are possible in this case. Firstly, the forward chemical reaction is of first order but the back-reaction is of second order, *viz.*,



(43)

Secondly, the forward chemical reaction is bimolecular, but the back-reaction is monomolecular which is illustrated by scheme (44):



(44)

The third possibility is at hand when both the forward and the reverse chemical reactions are bimolecular which, however, is a rare situation.

The steady state treatment of these schemes becomes hopelessly involved and hence only simplified cases will be considered below.

Employing Nernst relationship, the equation of the current-voltage curve for scheme (43) can be obtained in the following manner: As usual $A^\circ = (i_d - i)/k'_A$, and $i = k'_R\,(B^\circ + C^\circ)$. Assuming that the chemical reaction also is at all times in equilibrium and using homogeneous rate constants one has

$$i = k'_R\,(B^\circ + \sqrt{B^\circ k_3/k_4}) \tag{45}$$

from which, assuming further that $B^\circ \ll \sqrt{B^\circ k_3/k_4}$ it is found that

$$E = E' + \frac{RT}{nF}\ln\left[\frac{i_d - i}{i^2}\cdot\frac{(k'_R)^2}{k'_A}\cdot\frac{k_3}{k_4}\right] \tag{46}$$

In certain cases the concentration of D is approximately proportional to the current: $i = k'_R D^\circ$ and equation (46) can be written

$$E = E' + \frac{RT}{nF}\ln\left[\frac{i_d - i}{i}\cdot\frac{k'_R}{k'_A}\cdot\frac{k_3}{k_4}\right] - \frac{RT}{nF}\ln D^\circ \tag{47}$$

The constancy of the concentration of D at the surface of the electrode determines the wave form. Constant D° only gives a reversible wave, otherwise it will have irreversible characteristics, *i. e.*, owing to the proportionality between the current and D° the log plot slope will be non-linear. Scheme (43) represents a typical situation in which a metal complex is reduced to another oxidation state and the complexing agent is liberated.

Turning now to scheme (44) the current-voltage relationship is found in a similar fashion. Again $A^\circ = (i_d - i)/k'_A$ but now the current is proportional to the sum of the concentrations of B and D, *i. e.*, $i = k'_R\,(B^\circ + D^\circ)$. Assuming also here that the chemical equilibrium is attained instantaneously one has

$$B^\circ = \frac{i}{k'_R\left(1 + \frac{C^\circ k_3}{k_4}\right)} \tag{48}$$

and thus

$$E = E' + \frac{RT}{nF} \ln \left[\frac{i_d - i}{i} \cdot \frac{k'_R}{k'_A} \cdot \left(1 + C^\circ \frac{k_3}{k_4} \right) \right] \tag{49}$$

Depending on the relative magnitudes of k_3, k_4 and the surface concentration of C, different waves are found. First of all, if k_4 is very large, *i. e.*, B hardly decomposes at all, then the wave is reversible; $C^\circ k_3/k_4 = 0$. The case can be that $C^\circ k_3/k_4 \gg 1$, then depending upon the constance of C° during the electrolysis, the wave can show reversible characteristics (constant C°, high concentration of buffer or complexing agent) or can appear more or less irreversible (C° decreases on going up along the wave, *e. g.*, inadequate buffer capacity or slow rate of proton splitting). The half-wave potential will thus be a function of $(1 + C^\circ k_3/k_4)$ giving a number of various situations.

When the bimolecular chemical reaction is irreversible, steady state treatment can be applied. Consider the following, somewhat simplified reaction scheme in terms of the heterogeneous rate constants:

$$A \underset{}{\overset{k_A}{\rightleftharpoons}} A^\circ + ne^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B^\circ + C^\circ \xrightarrow{k_3} D^\circ \qquad B^\circ \xrightarrow{k_B} B \tag{50}$$

Assuming a steady state to be attained, one has

$$Ak_A + B^\circ k_2 = A^\circ k_A + A^\circ k_1 \tag{51}$$

$$A^\circ k_1 = B^\circ (k_2 + k_3 + C^\circ k_3) \tag{52}$$

From equations (17), (51) and (52) then

$$\frac{i_d - i}{i} = \frac{k_A}{k_1} \left[\frac{k_2 + k_B + C^\circ k_3}{k_B + C^\circ k_3} \right] \tag{53}$$

It is readily seen that when $k_2 \gg k_B + C^\circ k_3$, a reversible polarographic wave is found, otherwise it will be irreversible. As to the effect of the variation of the surface concentration of C, the same applies as was said above in connection with the reversible cases.

Numerous polarographic processes belonging to the group involving a follow-up bimolecular chemical reaction have been reported in the literature. Especially organic reductions and oxidations coupled with hydrogen ion uptake have been analyzed. Reference is made to the section of organic polarography in the monograph on polarography by Kolthoff and Lingane [16].

CONCLUSION

The results of the theoretical considerations in this paper can be summarized as follows:

1. Equation (20) represents a general relationship from which current-voltage equations can be derived for polarographic processes coupled with a

monomolecular chemical reaction. Equation (21) is valid under normal polarographic conditions and illustrates that $E_{\frac{1}{2}}$ will always be independent of the concentration of the reactant.

A *reversible* wave is found when

a) the chemical reaction is *reversible* and either of conditions (22) or (27) is satisfied;
b) the chemical reaction is *irreversible* and either of conditions (36) or (39) is fulfilled.

An *irreversible* wave is the result when

c) the chemical reaction is *reversible* and $k_2 \sim k_3 \gg k_4$;
d) the chemical reaction is *irreversible* and $k_2 \leq k_3$;
e) in all cases where k_1, $k_2 \leq k_A$, k_B, k_C or when $k_1 \gg k_2$.

2. Where a *bimolecular* chemical reaction affects the concentration of the product of the electron transfer reaction, the same general principles apply as in the previous case, except that here a situation corresponding to conditions (27) does not in general produce a reversible wave. This class is characterized by the fact that $E_{\frac{1}{2}}$ is a function of the concentration of either reactant A or C.

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The Oslo Electron Diffraction Units for Gas Work

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A new electron diffraction sector apparatus, primarily intended for gas work and now in use for some time is described. The high tension source, electron gun and magnetic lens are of commercial type, the rest of the apparatus has been designed and constructed in our institute. The new apparatus has made it possible to increase the accuracy of the intensity measurements and to extend the intensity measurements far beyond the s-range earlier investigated. Besides the advantages thus opened with respect to precise measurements of molecular parameters it has become possible to increase very markedly the resolution power of the procedure.

The use of a rotating sector to compensate for the steep fall in the background of electron diffraction diagrams from a gas was suggested independently by Trendelenburg[1], Finbak[2] and P. P. Debye[3] in the thirties. In Oslo, the method has been in use since 1940, and the advantage of the method as compared to the visual method has since then been fully realized. A series of the problems studied in our laboratory, particularly those involving the difference procedure, would in our opinion hardly have been solved with the use of the visual method[4–15].

The first sector electron diffraction apparatus in Oslo was designed by Chr. Finbak in 1936. As this apparatus has earlier been described[16,17], and since the main principles are very much the same as for that recently designed by Brockway and Bartell[18], it will only be mentioned briefly in the present report.

The usefulness of the old apparatus may perhaps most easily be demonstrated by reproducing a photometer curve of a diagram of a comparatively large molecule. In Fig. 1 is presented, therefore, a photometer curve of *sym.* triphenylbenzene.

One of the main differences between this apparatus and that of Brockway and Bartell is the mounting of the sector. A central axle is applied in the Oslo apparatus instead of the peripherally mounted ball-bearing system. We have found the central axle simple and convenient and have, therefore, applied this type of construction also in the new apparatus. The electron source of the old

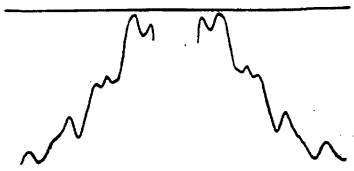

Fig. 1. Photometer record of sym. *triphenylbenzene from a diagram taken in the old sector apparatus.*

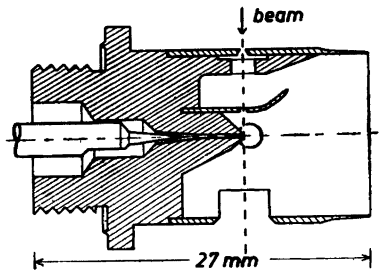

Fig. 2. Gas nozzle with needle valve used in the old apparatus (simplified).

apparatus consists of a cold-cathode discharge tube. The high voltage supply is also an old-fashioned construction but gives a stable direct current.

In order to secure the best possible construction for the new apparatus many of the details were first tried out in the old unit. For instance, a whole series of different gas-nozzle designs were tried out. A nozzle type which has been much used is illustrated in Fig. 2. Various liquid-air traps have been tried and also different sector shapes and sector screening factors. Most of the diagrams of the old apparatus were taken with an s^2 or s^3 sector.

DESCRIPTION OF THE NEW OSLO APPARATUS

The new apparatus was designed in collaboration with Professor Chr. Finbak (†). The ideas leading to many of the improvements we owe to him. The apparatus has to a great extent been built in the workshop of our institute. Exceptions are the high voltage supply and the electron gun which were purchased from Metropolitan-Vickers, England. This is standard equipment for an electron microscope. Only one magnetic lens is used for focussing the beam.

Fig. 3 is a simplified sketch and Fig. 4 a photograph of the apparatus. As may be seen from the scale, the dimensions are larger than for previously-built diffraction units. We chose this size in order to be able to place various kinds of auxiliary equipment inside the vacuum system and to avoid limitation in scattering angles due to the geometry of the apparatus. The various parts of the apparatus are fitted together with O-rings. O-rings are also used for the windows and the plate-box door.

The vacuum chamber consists of two steel cylinders, the inner diameters of which are 50 and 63 cm, respectively. The total inner height is 80 cm and the volume approximately 180 liters. The inner edge of the (shelf-like) connection between the cylinders is supported by steel pillars.

The main opening giving access to the chamber is a door (30×13 cm^2) in the bottom cylinder. Through this door the plate box may be inserted when the carriage is in its lowest position. In the top and bottom plates of the chamber and in the side walls of the upper cylinder a series of openings is present. Thus, one may introduce electron gun, pumps, gauges, cooling trap and various

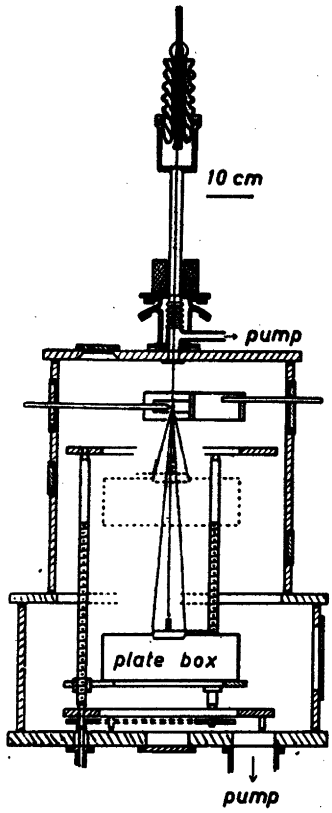

Fig. 3. Simplified section sketch of the new diffraction apparatus.

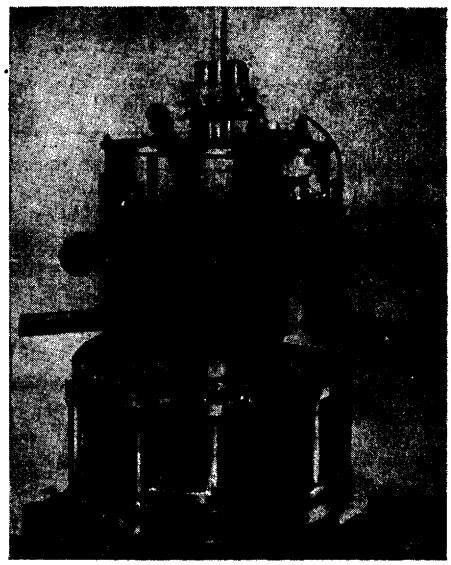

Fig. 4. Photograph of the main part of the new apparatus.

auxiliary equipment. Plexiglas windows are provided. Four standard sizes for the openings have been chosen, thereby allowing the same pieces of equipment to be moved from one position to another. This, of course, adds to the flexibility of the whole arrangement.

The plate box. The capacity of the plate box (Fig. 5) is 15 plates or more of maximum size 6 × 24 cm². The box is mounted on tracks and the loading or disloading is therefore very easy. The unexposed plates are stored in the first of three compartments. By means of a chain driven system operated by one single control, the plates may be transferred to the second compartment for exposure, and after exposure to the third compartment. The same control also opens and closes the exposure lid. In the exposure compartment, the two ends of the plates are pressed upwards against slide rails in the top of the box, the emulsion side thus being brought in a well-defined position. The plate-box mounting can be moved up and down and the distance between the photographic emulsion and the diffraction point thus be continuously varied. In order to keep the plate box exactly horizontal during this movement, the lift is

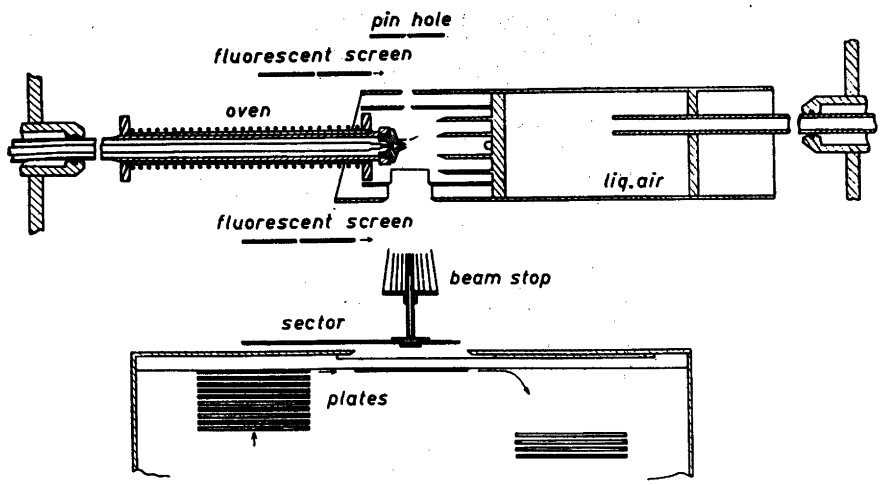

Fig. 5. Section through oven, liquid-air trap, and plate box with sector and beam stop (simplified).

operated by three screws which are rotated simultaneously by a chain connecting the toothed wheels at the end of each screw. The lift can be operated from the outside of the apparatus by means of one of the vacuum-tight shaft-bearings. By simply choosing the proper windows for nozzle and the liquid-air trap it is possible to vary the distance between the photographic plate and the diffraction point from a few centimeters to about half a meter.

The sector should be kept in a position close to the photographic plate although the nozzle-to-plate distance can be changed within wide limits. The sector mount, therefore, forms part of the carriage system. If necessary, however, it may be unfastened by a single hand operation. Gear transmissions, intended for rotation of the sector, as well as for adjustment in two horisontal directions, have been provided. If desired, the sector may also be moved out of the plate area. The carriage movements do not interfere with the operation of the sector or the plate box.

The sector found to be most satisfactory at the present time has a spiral form as shown in Fig. 6. Within the greater part of the s-interval the screening factor is approximately proportional to s^3; in the inner part, the opening is made larger than corresponds to this function. The sector is made from a phosphorus-bronze sheet of thickness 0.2 mm. To avoid deviation from planarity the sector is supported by duraluminium edges on both sides. The sector is operated by an electric motor placed outside the apparatus. The shape of the sector has been carefully studied both by direct measurement under the microscope and by diffraction experiments. From these studies, sector correction curves for use in our structure-determination work have been computed. The beam stop, mounted on the top of the sector, consists of a series of concentric cylinders. The beam stop used for the short-distance pictures, where we are mainly interested in the largest s-values, consists of seven cylin-

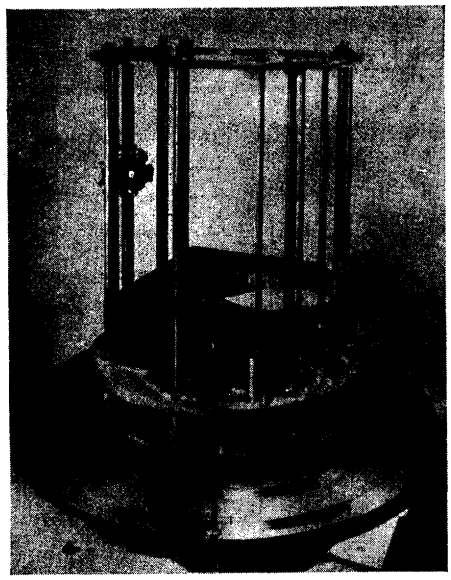

Fig. 6. Photograph showing the carriage arrangement. Sector replaced by a paper model.

ders. In the bottom of the beam stop is placed a layer of carbon black, which has a very low secondary electron emission. Colloidal graphite suspension can be used for coating the cylinder walls. The use of an effective beam stop seems to be of the greatest importance in order to obtain reliable intensity data at large s-values.

The nozzle and liquid-air trap designs seem also to be of great importance for the quality of the diffraction diagrams. Several oven- and nozzle types for various purposes have been constructed. The nozzle and the trap are placed on opposite sides of the apparatus, but at the same height. The end of the trap is a cylinder surrounding the nozzle. In this way, practically all the gas leaving the nozzle is condensed in the first trap and is accordingly prevented from reaching the rest of the apparatus. In Fig. 5, a typical nozzle-trap combination is shown.

The new apparatus has enabled us to enlarge our s-interval considerably. In the old apparatus we were able to obtain intensity values ranging from $s = 3$ Å^{-1} to nearly $s = 30$ Å^{-1}. The new apparatus gives intensity values extending from s equal to 1 Å^{-1} to s-values of about 60 Å^{-1}. Experimental curves giving intensity values over a large s-range are reproduced in Fig. 7. Corresponding theoretical curves are also given, for the sake of comparison. In our standard procedure we usually limit ourselves to three different distances between diffraction point and photographic plate, *viz.* approximately 49 cm, 19 cm and 12 cm. The experimental intensity curves like those reproduced in Fig. 7 are obtained in the usual way: A Leeds and Northrup microphotometer is used, and the plates are oscillated during the process of recording. The

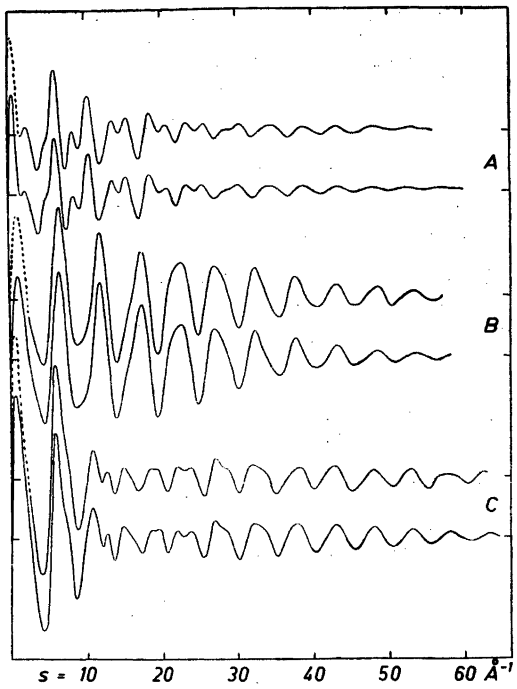

Fig. 7. Experimental and theoretical intensity curves of cyclooctatetraene *(A), NO_2 (B), and vinylacetylene (C).*

photometer curves are read off in a logarithmic scale and the blackness is transferred into intensity values before the theoretical background is subtracted.

The wavelength control is based upon diffraction pictures from gold foils or from evaporated gold films. Other reference substances have been tried, but at present we feel that gold gives the most reproducible values. An *s*-sector is used when taking calibration pictures. Without a sector, a slight decrease in wavelength with increasing *s*-values is observed. This decrease is probably due to electric charging effects in the photographic plates.

As we have found our present commercial high-voltage meter not to be sufficiently accurate for precision determinations we are building a new instrument for high voltage control. This outfit has, however, not yet been in operation and it will not be described here.

The apparatus has been briefly described by one of us in a lecture given at the Eighth Scandinavian Chemistry Congress in Oslo, June 1953 [19].

We want to express our sincere gratitude towards *Norges Teknisk-Naturvitenskapelige Forskningsråd* and *Norges almenvitenskapelige forskningsråd* for grants enabling us to build the new unit and use it for structure determinations. We are also very much indebted to Mr. S. Sørensen, senior instrument maker in our institute, for his excellent contribution to the construction work, and to our colleague cand. real. A. Almenningen

for his most valuable help in setting up the apparatus and his initiative and skill during the running-in period. Very helpful suggestions regarding technical improvements are due to him. We also want to express our gratitude to instrument maker Th. Nilsen, Trondheim, for having taken care of the construction of the plate box.

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Versuche zur Darstellung optisch aktiver bororganischer Verbindungen. II.* Über Ester der Diarylborsäuren

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Arylborsäuredibutylester reagieren mit Arylmagnesiumbromid, Molverhältnis 1 : 1, bei tiefer Temperatur (−60° bis −65°) unter Bildung von Diarylborsäureester. Borverbindungen, zwei verschiedene Arylgruppen enthaltend, wurden dargestellt.

Vertreter der Diarylborverbindungen sind schon von Michaelis [1], König und Scharrnbeck [2] sowie von Mel'nikov [3,4] dargestellt worden. Nach Michaelis erhält man Diphenylborchlorid durch Einwirkung von Phenylbordichlorid auf Quecksilberdiphenyl:

$$C_6H_5BCl_2 + Hg(C_6H_5)_2 \rightarrow (C_6H_5)_2BCl + C_6H_5HgCl$$

König und Scharrnbeck stellten Diphenylborsäure in folgender Weise dar:

$$2\ C_6H_5MgX + B(OR)_3 \rightarrow (C_6H_5)_2BOR + 2\ ROMgX \xrightarrow{\text{Hydrolyse}} (C_6H_5)_2BOH$$

Torssell [5] hat einen Weg zur Darstellung von Diarylborverbindungen mit zwei verschiedenen Gruppen angegeben. Wenn eine Grignardverbindung bei tiefer Temperatur (—60° bis —65°) zu der ätherischen Lösung von einem Monoarylborsäureester im Molverhältnis 1:1 hinzugefügt wird, lässt sich die Reaktion auf der Stufe der Diarylborverbindung halten:

$$Ar'MgX + ArB(OR)_2 \rightarrow Ar'ArBOR \xrightarrow{\text{Hydrolyse}} Ar'ArBOH$$

Der bei der Grignardierung entstandene Diarylborsäureester lässt sich nicht aus der Lösung herausdestillieren, denn er wird von den Magnesiumsalzen infolge Komplexbildung festgehalten:

$$Ar'ArBOR + ROMgX \rightarrow [Ar'ArB(OR)_2]^- MgX^+$$

Das Reaktionsprodukt wird deswegen in verdünnter Schwefelsäure hydrolysiert. Die ätherische Schicht wird abgetrennt und mit etwas Wasser ge-

* I. *Acta Chem. Scand.* 8 (1954) 1779.

Tabelle 1.

Verbindung	Molekulargewicht	Siedepunkt °C	Siedepunkt Torr	Brechungsindex n_D^{25}	Dichte d_4^{25}	Molekularrefraktion	Atomrefraktion des Bors	Ausbeute %
o-Tolyl-*m*-tolyl-borsäure-*n*-propylester	252,2	173–5	11	1,5407	0,9646	82,12	5,43	53
o-Tolyl-*p*-tolyl-borsäure-*n*-propylester	252,2	173–5	10	1,5420	0,9648	82,27	5,28	53
o-Tolyl-phenyl-borsäure-*n*-propylester	238,2	156–9	9	1,5440	0,9749	77,10	5,10	41
o-Tolyl-*p*-chlor-phenyl-borsäure-*n*-propylester	272,6	176–7	10	1,5522	—	—	—	70

waschen. Nach dem Abdestillieren des Äthers wird die Diarylborsäure mit Propylalkohol verestert.

Kürzlich sind einige Arbeiten von Letsinger und Mitarbeitern [6,7] erschienen, die unabhängig von uns diese Methode ausgearbeitet haben. Es wird z. B. die Darstellung von Phenyl-α-naphtylborsäure-aminoäthylester beschrieben.

Die Diarylborsäurepropylester bilden farblose, feuchtigkeitsempfindliche Flüssigkeiten, die an der Luft allmählich bräunlich werden. Sie sind in organischen Lösungsmitteln stark löslich, dagegen in Wasser unlöslich. In Tabelle 1 sind ihre physikalischen Daten zusammengestellt. Wegen der Luftempfindlichkeit der Substanzen werden sämtliche Arbeitsoperationen unter Stickstoff durchgeführt.

Die Boranalysen sind nach der Methode von Wittig und Mitarbeitern [8] und Torssell [5] vorgenommen worden.

EXPERIMENTELLER TEIL

o-Tolyl-p-tolylborsäure-n-propylester. Ein 100 ml Kolben mit seitlichem Zuleitungsrohr für Stickstoff wurde wiederholt evakuiert und mit Stickstoff gefüllt. 9,5 g *p*-Tolylborsäuredibutylester [5] in 10 ml Äther wurden vorgelegt und auf −65° bis −60° gekühlt. Diese Temperatur wurde während der Reaktion beibehalten. 45 ml 1,05 *M* *o*-Tolylmagnesiumbromid-Lösung wurden unter Schütteln tropfenweise zugesetzt (10 % Überschuss). Man liess dann die Lösung langsam über Nacht Zimmertemperatur erreichen. Dabei entstand ein weisser Niederschlag. Die Magnesiumsalze wurden unter Stickstoff in 50 ml Eiswasser, das mit 2 ml konz. Schwefelsäure versetzt war, hydrolysiert. Die ätherische Schicht wurde abgetrennt und zweimal mit 30 ml Wasser gewaschen. Der Äther wurde bei gewöhnlichem Druck abdestilliert. 15 ml Wasser wurden zugegeben, worauf das Butanol-Wasser-Gemisch im Vakuum abgetrieben wurde. Die Badtemperatur wurde auf 75° gesteigert. Im Kolben blieb die Diarylborsäure als ein Öl zurück. 10 ml *n*-Propylalkohol wurden zugegeben, und aus der Mischung wurde zunächst über eine kleine Kolonne das konstant siedende Propanol-Wasser-Gemisch herausdestilliert. Nach dem Abdestillieren des Überschusses von Propylalkohol wurde der Rückstand im Vakuum gleichfalls über einer kleinen Kolonne destilliert. Folgende Fraktionen wurden bei 11 Torr gewonnen:

I.	→173°	0,5 ml
II.	173–176°	0,6 ml
III.	176–178°	6,0 ml

Fraktion III wurde noch einmal destilliert, wobei 5,1 g (53 %) *o*-Tolyl-*p*-tolyl-borsäure-*n*-propylester, Kp. 173—5°/10 Torr gewonnen wurden. (Gef. B 4,25. Ber. für $C_{17}H_{21}OB$ (252,2): B 4,29).

o-Tolyl-phenylborsäure-n-propylester wurde aus Phenylborsäuredibutylester [5] und *o*-Tolylmagnesiumbromid nach der oben beschriebenen Methode dargestellt. Kp. 156—9°/9 Torr. Ausbeute 41 %. (Gef. B 4,64. Ber. für $C_{16}H_{19}OB$ (238,2): B 4,54).

o-Tolyl-m-tolylborsäure-n-propylester aus *o*-Tolylborsäuredibutylester [5] und *m*-Tolylmagnesiumbromid. Kp. 173—5°/11 Torr. Ausbeute 53 %. (Gef. C 80,83; H 8,80; B 4,28. Ber. für $C_{17}H_{21}OB$ (252,2): C 80,97; H 8,39; B 4,29).

o-Tolyl-p-chlor-phenylborsäure-n-propylester aus *o*-Tolylborsäuredibutylester und *p*-Chlor-phenylmagnesiumbromid. Kp. 176—7°/10 Torr. Ausbeute 70 %. (Gef. C 71,41; H 6,79; Cl 12,68; B 4,05. Ber. für $C_{16}H_{18}OClB$ (272,6): C 70,50; H 6,65; Cl 13,00; B 3,97).

ANALYTISCHES

Da die Diarylborsäuren in Wasser unlöslich sind, wird die abgewogene Probe (~ 50 mg) in 6 ml Methanol gelöst. Unter Umschwenken werden 10 ml von einer bei Zimmertemperatur gesättigten wässrigen Quecksilberdichlorid-Lösung langsam zugetropft. Bei schnellem Zusatz scheidet sich die Diarylborsäure als ein Öl aus und wird vom Quecksilberdichlorid unvollständig angegriffen. Die quecksilberorganische Verbindung fällt allmählich aus. Man fügt 15 ml Wasser hinzu und lässt die Lösung etwa 30 Min. stehen. Natriumchlorid wird zugesetzt, worauf man mit 0,1 *N* Natriumhydroxyd-Lösung in Gegenwahrt von Methylrot und Phenolfthalein titriert. Bei der ersten Titrierung gegen Methylrot kommt es oft vor, dass der Umschlag nicht ganz stabil ist. Dies beruht darauf, dass die Umsetzung von Quecksilberdichlorid mit der Organoborverbindung noch nicht ganz beendet ist. Man setzt dabei den Natriumhydroxydzusatz so lange fort, bis die Lösung nach 5 Min. Stehen ihre blassgelbe Umschlagfarbe beibehält.

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Versuche zur Darstellung optisch aktiver bororganischer Verbindungen. III.* Über Ester der Aryl-alkylborsäuren

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Arylborsäuredialkylester setzen sich mit Alkylmagnesiumbromid, Molverhältnis 1 : 1, bei tiefer Temperatur (−60° bis −65°) unter Bildung von Aryl-alkylborsäureester um. Wenn die Grignardverbindung im Molverhältnis 2 : 1 dem Arylborsäuredialkylester zugesetzt wird, entsteht nicht das erwartete Dialkyl-arylborin, sondern es tritt eine Disproportionierung ein. Es bilden sich Trialkylborin und Triarylborin.

Durch Umsetzung von Phenylboroxyd mit Äthylmagnesiumbromid versuchten Krause und Nobbe [1] Phenyl-diäthylborin darzustellen. Die Reaktion verlief aber nicht in der erwarteten Richtung:

$$C_6H_5B{=}O + 2\ C_2H_5MgBr \rightarrow C_6H_5B(C_2H_5)_2 + (MgBr)_2O,$$

sondern es entstand eine Mischung von Triäthylborin und Triphenylborin. Phenyl-diäthylborin disproportioniert sich nach folgender Gleichung:

$$3\ C_6H_5B(C_2H_5)_2 \rightarrow (C_6H_5)_3B + 2\ (C_2H_5)_3B.$$

In der vorliegenden Arbeit wurde die Reaktion zwischen Phenylborsäuredialkylester und Alkylmagnesiumbromid studiert. Der Befund von Krause konnte bestätigt werden. Bei der Umsetzung von Propylmagnesiumbromid mit Phenylborsäuredipropylester (Molverhältnis 2:1) wurde Tripropylborin in 88 %-iger Ausbeute gewonnen. Im Kolben blieb reines Triphenylborin zurück.

$$C_6H_5B(OC_3H_7)_2 + 2\ C_3H_7MgBr \rightarrow [C_6H_5B(C_3H_7)_2] \rightarrow (C_6H_5)_3B + 2\ (C_3H_7)_3B$$

Äthylmagnesiumbromid und Phenylborsäuredibutylester ergaben Triäthylborin. Es ist zu erwarten, dass Phenyl-diäthylborin einen Siedepunkt von etwa 80°/10 Torr haben soll (vgl. den Siedepunkt des Phenyl-cyclotetrametylenborins [2]). Tatsächlich destillierte bei dieser Temperatur ein wenig Substanz über, die sich aber als sehr unrein erwies.

* II. *Acta Chem. Scand.* **9** (1955) 239.

Bei der Umsetzung von Phenyl-methyl-borsäurepropylester mit Methylmagnesiumbromid, Molverhältnis 1:1, konnte kein Phenyl-dimethylborin nachgewiesen werden.

Die Disproportionierung ist folgendermassen zu formulieren:

$$C_6H_5B(C_2H_5)_2 + C_2H_5MgX \rightarrow [(C_6H_5)\,B(C_2H_5)_3]^-MgX^+ \rightarrow C_6H_5MgX + (C_2H_5)_3B$$
$$C_6H_5MgX + C_6H_5B(C_2H_5)_2 \rightarrow [(C_6H_5)_2B(C_2H_5)_2]^-MgX^+ \rightarrow C_2H_5MgX +$$
$$+ (C_6H_5)_2B(C_2H_5) \text{ usw.}$$

Wenn man aber zum Phenylborsäuredibutylester das Grignardreagens im Molverhältnis 1:1 hinzufügt, erhält man in guter Ausbeute einen Phenylalkylborsäureester:

$$C_6H_5B(OC_4H_9)_2 + C_2H_5MgBr \rightarrow C_6H_5B\begin{matrix}C_2H_5\\OC_4H_9\end{matrix}$$

Diese Verbindung ist stabil und lässt sich unzersetzt destillieren. Eine Disproportionierung tritt also bei unseren Versuchsbedingungen nur ein, wenn alle drei Valenzen des Bors von Kohlenstoff besetzt werden. Die Ester der Monoaryl- und Diarylborsäuren [2,3] zeigen keine Neigung zur Disproportionierung.

Bei Phenyl-cyclopentamethylenborin und Phenyl-cyclotetramethylenborin [2] liess sich keine Disproportionierung feststellen. Diese wird durch die Ringkonfiguration verhindert.

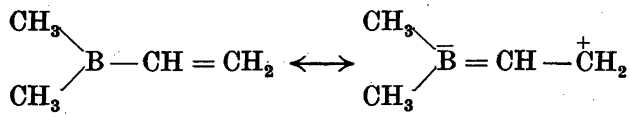
Über unsere Versuche asymmetrische Triarylborine darzustellen, soll in der nächsten Arbeit dieser Reihe berichtet werden. Die Arylverbindungen des Bors sind im allgemeinen stabiler als die Alkylverbindungen; man muss aber trotzdem mit der Möglichkeit einer Disproportionierung rechnen. Wenn diese eintritt, ist es auf diesem Wege unmöglich, optisch aktive Organoborverbindungen darzustellen.

Die Versuche von Johnson und Mitarbeitern [4] sowie von Rohtstein und Saville [5] asymmetrische Trialkylborine darzustellen, sind gescheitert. Parsons und Ritter [6] fanden, dass ungesättigte, asymmetrische Trialkylborine eine unerwartete Stabilität zeigen. Dimethyl-vinylborin disproportioniert sich nur langsam bei Zimmertemperatur. Die Disproportionierung vollzieht sich anscheinend ohne Einfluss von Lösungsmittel oder Metall-alkyl.

Die einfachste Bor-wasserstoffverbindung, BH_3, und die von Schlesinger und Mitarbeitern [7,8] dargestellten Alkyl-borhydride, die zur Disproportionierung leicht befähigt sind, liegen in ihrer dimeren Form vor. Dagegen entspricht die Gasdichte des Trimethylborins [9] der einfachen Formel $B(CH_3)_3$. Wenn man aber eine geringe Neigung zur Dimerisierung annimmt, könnte dies die Disproportionierung der asymmetrischen Trialkylborine erklären.

$$2\ R'R''_2B \rightarrow \left[\begin{matrix}R'' & & R'' \\ & B\quad B & \\ R' & & R''\end{matrix}\right] \rightarrow R''_3B + R'_2R''B$$

Bei Dimethyl-vinylborin haben wir mit zwei mesomeren Strukturen zu rechnen:



was die Disproportionierung erschwert.

Methyl-dibutylborin lässt sich aus Natrium(Kalium)-dibutylborid und Methyljodid darstellen [10]. Über die Stabilität der Verbindung wurden keine Angaben gemacht.

Die Aryl-alkylborsäureester lassen sich auch nach der Sublimat-Methode analysieren; die Reaktionsverhältnisse sind aber mehr kompliziert. Bei der Analyse von Phenyl-äthylborsäurebutylester wurde $HCl:H_3BO_3$ gleich 1:1 gefunden; das heisst, Quecksilberdichlorid zersetzt nicht die B—C_{aliph}-Bindung. Bei Phenyl-methylborsäurepropylester wurde das Verhältnis als 1:2 gefunden. Dies kann nur so erklärt werden, dass, erst wenn die Lösung bei der Titrierung etwas alkalisch wird, die Spaltung von der B—C_{aliph}-Bindung durch Einwirkung von Quecksilberdichlorid einsetzt. Tri-*n*-propylborin wird von Quecksilberdichlorid in Methanol-Wasser-Lösung äusserst langsam angegriffen.

EXPERIMENTELLER TEIL

Umsetzung von Phenylborsäuredi-n-propylester mit n-Propylmagnesiumbromid im Molverhältnis 1 : 2. In einem Kolben wurden zu 7,3 g Phenylborsäuredi-*n*-propylester in 5 ml Äther unter Stickstoff und Kühlung mit kaltem Wasser 75 ml 1,10 *M* *n*-Propylmagnesiumbromid-Lösung zugetropft (15 % Überschuss). Zwei Schichten bildeten sich. Während des Zusatzes schüttelte man kräftig um. Die Wärmetönung bei der Reaktion war gering. Die Mischung wurde eine halbe Stunde auf dem Wasserbad unter Rückfluss gekocht, worauf sie in 40 ml Eiswasser unter Stickstoff hydrolysiert wurde. Die ätherische Schicht wurde abdekantiert, und die ausgefällten Magnesiumsalze wurden mit 15 ml Äther gewaschen. Von den vereinigten Ätherlösungen wurde der Äther nach Trocknen über Calciumchlorid bei gewöhnlichem Druck abdestilliert. Bei 90 bis 100° destillierte ein wenig *n*-Propylalkohol und Wasser über. Der Rückstand im Kolben wurde im Vakuum destilliert. Es wurden bei 51—3°/10 Torr 2,9 g Tri-*n*-propylborin (60°/20 Torr) [11] gewonnen. Ausbeute 88 %. Im Kolben blieb Triphenylborin zurück, das beim Erkalten in langen weissen Prismen kristallisierte. Ein Tropfen Tri-*n*-propylborin, der auf einem Uhrglas in Berührung mit der Luft gebracht wurde, erstarrte nach kurzer Zeit und ergab blätterförmige Kristalle von *n*-Propylborsäure [11]; Schmp. 107°.

Umsetzung von Phenylborsäuredi-n-butylester mit Äthylmagnesiumbromid im Molverhältnis 1 : 2. Nach der oben beschriebenen Methode wurden 11,1 g Phenylborsäuredi-*n*-butylester mit 100 ml 1,02 *M* Äthylmagnesiumbromid-Lösung (10 % Überschuss) versetzt. Nach Hydrolysieren und Trocknen der Ätherlösung mit Calciumchlorid wurde der Äther abdestilliert. Mit dem Äther destillierte eine borhaltige Substanz über. Zwischen 80 und 100° entwich noch mehr davon zusammen mit etwas Butylalkohol und Wasser. Beim Verdampfen des Destillates auf einem Uhrglas an der Luft blieb eine weisse Substanz zurück, die als Borsäure identifiziert wurde. Triäthylborin siedet bei 95°/760 Torr und entzündet sich von selbst an der Luft [9]. Bei der nachfolgenden Vakuumdestillation wurden bei 10 Torr folgende Fraktionen gewonnen:

I.	30— 40°	2,0	ml
II.	40— 75°	0,1	»
III.	75— 90°	0,8	»
IV.	90—110°	0,3	»
V.	110—115°	0,7	»

Sämtliche Fraktionen waren farblos. Im Kolben blieb eine ölige, gelbe Flüssigkeit, ~ 1,5 ml, zurück.

Die Fraktion I bestand aus *n*-Butylalkohol. III bildete eine unangenehm riechende Flüssigkeit, die wahrscheinlich etwas Phenyldiäthylborin enthielt. V wurde als Phenyl-äthylborsäure-*n*-butylester identifiziert.

Die Fraktion III wurde noch einmal destilliert aber ergab nur uneinheitliche Produkte. Dies könnte auf einen Zerfall der Substanz während der Destillation deuten.

Umsetzung von Phenylborsäuredi-n-butylester mit Äthylmagnesiumbromid im Molverhältnis 1 : 1. Darstellung von Phenyl-äthylborsäure-n-butylester. Zu 9,5 g Phenylborsäure-di-*n*-butylester wurden unter Stickstoff und gutem Schütteln 41 ml 1,02 *M* Äthylmagnesiumbromid-Lösung tropfenweise hinzugefügt (2 % Überschuss). Die Reaktionstemperatur wurde bei −60° bis −65° gehalten. Man liess dann die Lösung langsam Zimmertemperatur annehmen. In einem Scheidetrichter wurde die ätherische Lösung auf 40 ml Eiswasser + 1,5 ml konz. Schwefelsäure gegossen. Die ätherische Schicht wurde mit 30 ml Wasser gewaschen und mit Calciumchlorid getrocknet. Der Äther wurde abdestilliert und der Rückstand mit 15 ml *n*-Butylalkohol in gewöhnlicher Weise verestert. Nach Abtreiben des Überschusses von *n*-Butylalkohol wurde der Phenyl-äthylborsäure-*n*-butylester in Vakuum destilliert. Er bildet eine farblose, luftempfindliche, unangenehm riechende Flüssigkeit vom Kp. 110−1°/9 Torr. Ausbeute 4,5 g oder 58 %. $n_D^{25} = 1{,}4864$; $d_4^{25} = 0{,}8940$. (Gef. C 75,5; H 10,22; B 5,55. Ber. für $C_{12}H_{19}OB$ (190,1): C 75,8; H 10,07; B 5,69).

Phenyl-methylborsäure-n-propylester. Aus Phenylborsäuredi-*n*-propylester und Methylmagnesiumbromid im Molverhältnis 1 : 1. Kp. 87−9°/9 Torr. $n_D^{25} = 1{,}4880$; $d_4^{25} = 0{,}8985$. (Gef. C 73,6; H 9,17; B 6,75. Ber. für $C_{10}H_{15}OB$ (162,0): C 74,1; H 9,33; B 6,68).

Phenyl-methylborsäure entsteht bei der Hydrolyse von Phenyl-methylborsäure-*n*-propylester. Sie bildet eine farblose Flüssigkeit, die mit Wasserdampf flüchtig ist. Sie ist etwas löslich in Wasser.

Umsetzung von Phenyl-methylborsäure-n-propylester mit Methylmagnesiumbromid. 2,5 g Phenyl-methylborsäure-*n*-propylester wurden mit 15 ml Methylmagnesiumbromid (7 % Überschuss) versetzt. Das Reaktionsprodukt wurde in der üblichen Weise aufgearbeitet. Es konnten Triphenylbor und eine leichtflüchtige, an der Luft selbstentzündliche Substanz (Trimethylborin ist ein Gas vom Kp. −20°) nachgewiesen werden. Phenyldimethylborin wurde nicht gewonnen.

Für die ökonomische Unterstützung dieser Arbeit bin ich *Edlunds Stipendiefond* zu besonderem Dank verpflichtet.

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2,4-Dihydroxyphenanthrene, a Phenanthrene Analogue of *cis*-Pinosylvin

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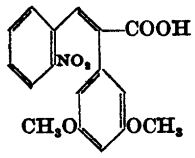
2,4-Dihydroxyphenanthrene has been prepared and was found to be less toxic than pinosylvin against certain wood-rotting fungi. The synthesis was performed according to the Pschorr procedure. The method of Cook and Hewett was not found to be satisfactory in this particular case.

Pinosylvin, 3,5-dihydroxy-*trans*-stilbene, and its monomethylether are the main toxic principles of the heartwood of pines. Their physiological properties have been studied by several authors[1-6]. 2,4-Dihydroxyphenanthrene (III) embodies the skeleton of *cis*-pinosylvin (IV) and it was considered to be desirable to prepare this phenanthrene derivative in order to compare its toxicity against wood-rotting fungi with that of pinosylvin. Similar products obtained from cheap starting materials such as resin acids might possess technical potentialities and it is interesting to note that several phenolic diterpenes have been isolated from the heartwood of various conifers [7].

2,4-Dihydroxyphenanthrene has been synthesised by a somewhat modified Pschorr procedure. 3,5-Dimethoxybenzoyl chloride was transformed by the Arndt-Eistert method into 3,5-dimethoxyphenylacetic acid (previously prepared by Mauthner [8] by a different route) and the latter compound was then condensed with *o*-nitrobenzaldehyde. The resulting *o*-nitro-α-(3,5-dimethoxyphenyl)-cinnamic acid (I) was reduced to the corresponding amino acid which after diazotisation and cyclisation yielded 2,4-dimethoxyphenanthrene-10-carboxylic acid (II). This compound, on decarboxylation followed by demethylation with pyridinium chloride, furnished 2,4-dihydroxyphenanthrene (III).

An attempt was also made to prepare 2,4-dihydroxyphenanthrene according to the method of Cook and Hewett. The crude tertiary alcohol from the reaction of 3,5-dimethoxy-β-phenyl-ethyl magnesium bromide with *cyclo*hexanone was cyclised by treatment with a sulphuric-acetic acid mixture and various dehydrogenation experiments were carried out on the product. In no case, however, could any recognisable products be isolated apart from un-

changed starting material. This result is almost certainly due to the fact that the spirane (V) and not the desired octahydro-phenanthrene (VI) is produced in the cyclisation reaction (*cf.* Cook *et al.*[9] and Barnes[10]).



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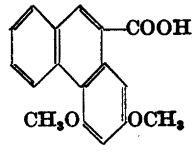

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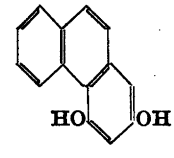
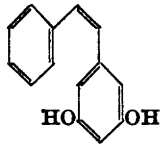
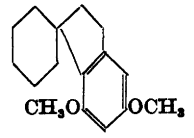


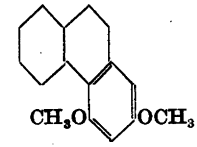
III



IV



V



VI

The toxicity of 2,4-dihydroxyphenanthrene and pinosylvin to some fungi has been studied by Docent E. Rennerfelt, Statens Skogsforskningsinstitut, Stockholm, to whom I am much indebted. In agar cultures no growth of the mycelia was observed with the following substances and organisms. Pinosylvin: 0.005 %, *Peniophora gigantea* and *Polyporus annosus;* 0.01 %, *Polyporus abietinus;* 0.02 %, *Fusarium culmorum* and *F. oxysporum.* (*Ophiostoma pini* was not inhibited at this concentration.) 2,4-Dihydroxyphenanthrene: 0.01 %, *Fusarium culmorum* and *Peniophora gigantea;* 0.02 %, *Fusarium oxysporum.* (*Ophiostoma pini, Polyporus abietinus* and *P. annosus* were not inhibited at this concentration.) It is thus apparent that 2,4-dihydroxyphenanthrene is a weaker fungicide than pinosylvin.

EXPERIMENTAL *

ω-Diazo-3,5-dimethoxyacetophenone. A solution of 3,5-dimethoxybenzoyl chloride (20 g) in absolute ether (300 ml) was slowly added with mechanical stirring to an excess of diazomethane in ether (prepared from 50 g of nitrosomethylurea in 600 ml of ether and dried over potassium hydroxide) at −5°. The solution was then allowed to stand for 4 hours, filtered from a small amount of a gummy impurity and evaporated *in vacuo* at about 30°. The yellow residue was dried in a vacuum desiccator. The diazo ketone could be crystallized from benzene-petroleum ether forming yellow needles melting at 72−73.5° without appreciable decomposition (the melt crystallized on cooling). At about 90°, however, decomposition took place. Yield 20.2 g. (Found: OCH_3 28.75. Calc. for $C_{10}H_{10}N_2O_3$ (206.2): OCH_3 30.01.)

ω-Chloro-3,5-dimethoxyacetophenone. To a solution of ω-diazo-3,5-dimethoxyacetophenone (200 mg) in ethanol (2 ml) was added conc. hydrochloric acid (0.3 ml). When the evolution of nitrogen had ceased the solution was heated and diluted with hot water. The chloro-ketone separated on cooling and was crystallized from ethanol (leaflets), m.p. 72.5−73.5°. (Found: Cl 16.5; OCH_3 29.13. Calc. for $C_{10}H_{11}O_3Cl$ (214.7): Cl 16.2; OCH_3 28.91.)

* All melting points uncorrected.

3,5-Dimethoxyphenylacetic acid. A solution of ω-diazo-3,5-dimethoxyacetophenone (20 g) in absolute ethanol (300 ml) was heated on a waterbath to 55—60° and silver oxide (from 30 ml of a 10 % silver nitrate solution) added in small portions with mechanical stirring during 3—4 hours. The solution was then boiled for one hour, filtered through a thin layer of animal charcoal and evaporated *in vacuo*. The residual ester was distilled yielding a colourless oil (17.4 g) b.p. 164—166°/8 mm. The ester was boiled for one hour with sodium hydroxide (100 ml: 2 *N*) and after cooling and filtering the solution was acidified and the precipitate collected (15 g) and crystallized from water. M.p. 102—103°, Mauthner [8] gives 99—100°.

o-Nitro-α-(3,5-dimethoxyphenyl)-cinnamic acid (I). A solution of 3,5-dimethoxyphenylacetic acid (12 g), *o*-nitro-benzaldehyde (9.5 g) and triethylamine (8.9 ml) in acetic anhydride (30 ml) was heated on a waterbath for 24 hours, water (6 ml) was carefully added and the heating continued for 0.5 hour. The mixture was then poured into dilute hydrochloric acid (500 ml) and the precipitated gum dissolved in dilute sodium hydroxide. After shaking with ether the alkaline solution was acidified giving a brownish yellow precipitate which was collected, dried and triturated with cold benzene (3 × 50 ml) giving a crystalline product. After one crystallization from benzene (50 ml) the yellow product (7.1 g) melted at 153—160°. It was used for the next step without further purification. After repeated crystallizations from ethanol the yellow prisms melted at 179.5—180.5°. (Found: OCH_3 18.93. Calc. for $C_{17}H_{15}NO_6$ (329.3): OCH_3 18.85.)

o-Amino-α-(3,5-dimethoxyphenyl)-cinnamic acid. The *o*-nitro-α-(3,5-dimethoxyphenyl cinnamic acid (6 g) was dissolved in aqueous ammonia (conc. ammonia 20 ml, water 30 ml) and the solution added to a hot mixture of ferrous sulphate (45 g $FeSO_4 \cdot 7\ H_2O$) and ammonia (conc. ammonia 160 ml, water 250 ml). The mixture was heated on a waterbath for 1.5 hours and after cooling the solution was filtered and carefully made slightly acid with hydrochloric acid. The greenish yellow precipitate (3.9 g) crystallized from ethanol in yellow prisms, m.p. 189—190°. (Found: OCH_3 20.39. Calc. for $C_{17}H_{17}NO_4$ (299.3): OCH_3 20.74.)

2,4-Dimethoxyphenanthrene-10-carboxylic acid (II). The *o*-amino-α-(3,5-dimethoxyphenyl)-cinnamic acid (3 g) was dissolved in sodium hydroxide solution (20 ml; 2 *N*) and precipitated with sulphuric acid (120 ml; 2 *N*). The mixture was cooled to about 0° and an aqueous solution of sodium nitrite (15 ml containing 0.8 g of sodium nitrite) was added dropwise with stirring. The stirring was continued for 4 hours and the excess of nitrite was then destroyed with urea. The yellow solution was filtered and the filtrate heated on a waterbath until it gave no colour reaction with α-naphthol. The precipitated acid was collected (2.6 g) and crystallized from ethanol (long needles), m.p. 225.5—226°. (Found: OCH_3 22.16. Calc. for $C_{17}H_{14}O_4$ (282.3): OCH_3 21.99.)

2,4-Dimethoxyphenanthrene. 2,4-Dimethoxyphenanthrene-10-carboxylic acid (2 g) was heated with Adkin's copper chromite catalyst (80 mg) in quinoline (10 ml). The evolution of carbon dioxide started at 210° and ceased after 1.5 hours at 220°. The mixture was cooled, poured into dilute hydrochloric acid and extracted with ether. The ethereal layer was washed successively with hydrochloric acid, dilute sodium hydroxide and water, dried over calcium chloride and evaporated. An oil was obtained which crystallised when triturated with a little methanol. The product (1.6 g) was distilled *in vacuo* and crystallized from ethanol (needles), m.p. 75.5—76.5°. (Found: OCH_3 25.8. Calc. for $C_{16}H_{14}O_2$ (238.3): OCH_3 26.05.)

2,4-Dihydroxyphenanthrene (III). 2,4-Dimethoxyphenanthrene (1 g) was heated with pyridinium chloride (3 g) at 180° for 2 hours and the mixture poured into dilute hydrochloric acid. A precipitate was formed which was dissolved in ether, washed with hydrochloric acid and filtered through aluminium oxide. After evaporation of the ether a light brownish residue (0.7 g) was obtained which was crystallized from water (animal charcoal) giving a slightly pink coloured product melting at 156.5—157°. (Found: C 80.35; H 4.79. Calc. for $C_{14}H_{10}O_2$ (210.2): C 79.98; H 4.79.)

2,4-Diacetoxyphenanthrene was prepared from 2,4-dihydroxyphenanthrene using acetic anhydride in pyridine. The product crystallized from ethanol in prisms, m.p. 136—137°. (Found: C 73.35; H 4.88. Calc. for $C_{18}H_{14}O_4$ (294.3): C 73.46; H 4.79.)

2,4-Dibenzoyloxyphenanthrene prepared from 2,4-dihydroxyphenanthrene by the Schotten-Baumann method crystallized from ethanol in prisms, m.p. 184—184.5°. (Found: C 80.23; H 4.38. Calc. for $C_{28}H_{18}O_4$ (418.4): C 80.37; H 4.34.)

3,5-Dimethoxy-β-phenylethyl alcohol. A solution of ethyl 3,5-dimethoxyphenylacetate (16 g) in absolute ether (100 ml) was slowly added with mechanical stirring to a slurry of lithium aluminium hydride (4 g) in dry ether (400 ml) at a rate sufficient to keep the solution boiling. The mixture was boiled for an additional 0.5 hour and the excess of hydride then destroyed by careful addition of water (15 ml) with cooling. Sulphuric acid (175 ml, 10 %) was added and the ethereal layer washed with water, dried over anhydrous sodium sulphate and the ether evaporated. The residue, distilled *in vacuo*, gave 3,5-dimethoxy-β-phenylethyl alcohol as a colourless oil (12 g), b.p. 126—130°/1—2 mm. The 3,5-dinitrobenzoate, prepared by heating the alcohol with 3,5-dinitrobenzoyl chloride in dry pyridine for a short time, crystallized from benzene-ligroin as yellow needles, m.p. 141.5—142°. (Found: OCH_3 15.33. Calc. for $C_{17}H_{16}N_2O_8$ (376.3): OCH_3 16.49.)

3,5-Dimethoxy-β-phenylethyl bromide. To a solution of 3,5-dimethoxy-β-phenylethyl alcohol (10 g) in carbon tetrachloride (20 ml) at 60° was added phosphorus tribromide (8 g) in one portion and the mixture refluxed for 45 minutes. The solution was washed with sodium carbonate solution followed by water, dried over anhydrous sodium sulphate and the solvent evaporated. The residue was distilled *in vacuo* yielding a colourless oil (8.2 g), b.p. 125—128°/1—2 mm. Redistillation followed by cooling of the distillate gave 3,5-dimethoxy-β-phenylethyl bromide as a solid, m.p. 20—22°. (Found Br 32.3; OCH_3 25.3. Calc. for $C_{10}H_{13}O_2Br$ (245.1): Br 32.6; OCH_3 25.3.) The bromide when refluxed with the silver salt of 3,5-dinitrobenzoic acid in dry benzene for 12 hours gave 3,5-dimethoxy-β-phenylethyl-3,5-dinitrobenzoate m.p. 144—145°, undepressed on admixture with a sample of the compound prepared from the alcohol as previously described.

1-β-(3,5-Dimethoxyphenyl)-ethylcyclohexanol. A mixture of 3,5-dimethoxy-β-phenylethyl bromide (7.5 g), magnesium turnings (0.8 g), a crystal of iodine and absolute ether (30 ml) was boiled until most of the metal had passed into solution. *Cyclo*hexanone (3.5 ml) in ether (10 ml) was then slowly added with cooling when a heavy oil separated. The reaction mixture was decomposed with ammonium chloride solution, the ethereal layer washed with sodium bisulphite solution followed by water and dried over anhydrous sodium sulphate. Evaporation of the ether followed by distillation gave 1-β-(3,5-dimethoxyphenyl)-ethyl*cyclo*hexanol (3.2 g) as a viscous, slightly yellowish oil, b.p. 165—170°/1—2 mm.

Cyclisation of 1-β-(3,5-dimethoxyphenyl)-ethylcyclohexanol. 1-β-(3,5-Dimethoxyphenyl)-ethyl*cyclo*hexanol (2 g) was refluxed for 1 hour with a mixture of glacial acetic acid (20 ml) and concentrated sulphuric acid (2 ml). The reaction mixture was poured into water, the product extracted with ether and the ethereal solution washed with sodium carbonate solution followed by water and dried. Evaporation of the ether gave a thick brownish yellow syrup containing some crystals. Distillation *in vacuo* (12 mm) afforded a colourless oil which immediately crystallized. Recrystallization from ethanol gave the substance as colourless prisms, m.p. 95—96°. (Found: C 78.0; H 9.07. Calc. for $C_{16}H_{22}O_2$ (246.3): C 78.01; H 9.00.)

Dehydrogenation experiments. (a) Sulphur. A mixture of cyclisation product (225 mg) and sulphur (115 mg) was heated to 210° when evolution of hydrogen sulphide commenced. After one hour at 220° the reaction mixture was distilled *in vacuo* but only a dark tar was obtained. A similar result was obtained when the reaction was carried out by heating *in vacuo* (200 mm) for one hour at 170° and then for an additional 0.5 hour at 200°.

(b) Selenium. Cyclisation product (200 mg) was heated with selenium (250 mg) at 290—300° for 12 hours. Hydrogen selenide was evolved but no recognisable product could be isolated from the reaction.

(c) Catalytic dehydrogenation. A 10 % palladium-carbon catalyst was employed under varying conditions — with the catalyst alone or with naphthalene or tetralin as solvent or with cinnamic acid as hydrogen acceptor. In all experiments either the substance was recovered unchanged or else intractable material was produced.

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The Conversion of Orotic Acid to Uridine Nucleotides *in vitro*

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The conversion of radioactive orotic acid to 5′-uridine monophosphate and three 5′-uridine pyrophosphate nucleotides was effected by dialyzed particle-free extracts from a number of animal and avian tissues. Ribose-5-phosphate, ATP and magnesium ion were required for the conversion. The conversion could also be obtained in extracts metabolizing hexose diphosphate. Chromatographic evidence indicated the 5′-uridine nucleotides to include uridine diphosphate and uridine triphosphate. Uridine and uracil were found not to be intermediates in the conversion although these compounds could be produced from the uridine nucleotides by enzymes present in the extracts.

By the use of a partially purified enzyme preparation from chicken liver it was demonstrated that ribose-5′-phosphate and ATP react to produce ribose triphosphate, presumably 5-phosphoribosyl pyrophosphate, and that this compound is the source of the ribose phosphate moiety of the uridine nucleotides. Ribose-1,5-diphosphate was inactive with this enzyme preparation, even in the presence of ATP. The 5′-uridine monophosphate was the first of the uridine nucleotides to be formed; the other nucleotides arose by the action of phosphorylating enzymes present in other enzyme fractions.

Recent evidence has demonstrated that orotic acid is a key intermediate in a series of metabolic reactions whereby the uracil of the polynucleotides is formed. Orotic acid was first found to be utilized as a specific precursor of the pyrimidines in the nucleic acids of the rat by Arvidson *et al.*[1] (*cf.* also [2]). It has since been shown to be a normal intermediate in the formation of the polynucleotides [3] and the individual steps in its enzymatic synthesis from aspartic acid, ammonia and carbon dioxide have been described [4–7]. In the liver of the living rat the compound was found to be converted almost quantitatively into the free uridine nucleotides ** which were apparently closely involved as pre-

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** 5′-Uridine phosphate (UMP), 5′-uridine diphosphate (UDP), 5′-uridine triphosphate (UTP) and several 5′-uridine pyrophosphoglycosyl compounds. Other abbreviations used in this paper are: 5′-adenosine nucleotides (AMP, ADP and ATP) and diphosphopyridine nucleotide (DPN).

cursors of the polynucleotide uracil [8]. The widespread occurrence of many enzymatic reactions of the uridine nucleotides has been the subject of numerous other reports (*cf.* Ref.[9]). Further detailed study of the reaction sequences involving orotic acid is of great importance in learning the overall mechanism of the formation of the polynucleotides.

In a continuation of the investigations previously carried out with living rats and rat liver slices, we have been engaged in studying the enzymatic processes by which the orotic acid is utilized. The object of this paper is to describe studies on the formation *in vitro* of the uridine nucleotides and to attempt to correlate the *in vivo* and *in vitro* processes. During the course of this project * the publications of Kornberg *et al.*[11,12] have appeared, which elucidate the enzymatic steps whereby orotic acid reacts with 5-phosphoribosyl pyrophosphate to form orotic acid ribotide and the subsequent decarboxylation of this product to form UMP. Our work has been done with different sources and procedures and is found to confirm and supplement that of Kornberg *et al.* A preliminary publication has appeared [13].

EXPERIMENTAL

Orotic acid-2-^{14}C containing about 1 μC per μmole was prepared by oxidation of $K^{14}CN$ to KCNO, by the method of Gall and Lohman [14] and the condensation of the KCNO with aspartic acid, by the method of Nyc and Mitchell [15].

Preparation of the particle-free extracts. The tissue was homogenized [10] in 4 volumes of 0.13 *M* KCl – 0.01 *M* $MgCl_2$ – 0.015 *M* potassium phosphate, pH 7.2, in a Potter-Elvehjem homogenizer and centrifuged at 60 000 – 70 000 g for 30 minutes in a Spinco centrifuge. Fat particles were partially removed by decantation of the supernatant solution through cotton or glass wool. The extracts were dialyzed against the same medium (50 ml of extract *versus* two successive 2 liter volumes) for 12 – 15 hours. All these operations were at 0 – 5° **.

Incubation. In the "hexose diphosphate system" the amounts of substrate used were: 0.5 μmole of orotic acid-2-^{14}C, 20 μmole of hexose diphosphate, 1.5 μmole of DPN, 120 μmole of nicotinamide and 2.0 ml of the tissue extract. In the "ribose phosphate system" the amounts used were: 0.5 μmole of orotic acid-2-^{14}C, 20 μmole of ribose phosphate, 20 μmole of 3-phosphoglyceric acid, 5 μmole of ATP and 2.0 ml of the tissue extract. In some experiments 3 mg of a muscle enzyme preparation [16] were also added to ensure utilization of the phosphoglyceric acid for regeneration of the ATP. The total volumes of both incubation systems were 4.0 ml. The incubations were carried out in 25 ml. Erlenmeyer flasks, which were shaken at 37° for 40 minutes.

At the end of the incubation period, the flasks were chilled and made to 0.4 *N* perchloric acid. The precipitated proteins were centrifuged and washed once with 1 ml of cold 0.2 *N* perchloric acid. The perchloric acid was neutralized in an ice bath with KOH, using phenol red as an internal indicator. The neutralized extract was decanted onto the chromatographic column through glass wool to remove the $KClO_4$ precipitate, which was washed once with cold water. The $KClO_4$ retained less than 2 % of the ^{14}C.

Chromatography. The principle of the gradient elution procedure previously described [17] was used although the technique was simplified to permit rapid routine work. Four columns, 6 cm × 1 cm, of Dowex-2 (formate) were eluted simultaneously by increasing concentrations of eluent from one 500 ml mixing flask, which received the

* The original observation of Saffran and Scarano [10], that 5-phosphoribose is utilized by pigeon liver extracts to convert adenine to AMP, provided a valuable stimulus for the work with orotic acid.

** The activity of the extract in the "hexose diphosphate system" diminished 20—40% during the dialysis. The larger losses were found at the higher temperatures and longer times of dialysis.

concentrated eluent by gravity flow from the reservoir flask. Fractions of 5 ml volume were collected by an automatic collector. The mixing flask initially contained water and the reservoir flask 6 *N* formic acid; after 25 fractions had been collected from each column (resulting in a concentration of formic acid in the mixing flask of about 4 *N*) the reservoir flask was changed to contain 4 *N* formic acid — 1.5 *M* ammonium formate. Another 25 fractions were collected so that the final concentration of eluent in the mixing flask was about 4 *N* formic acid — 1 *M* ammonium formate. All of the ^{14}C was eluted from the columns in this way.

The light absorption of the fractions was read at 260 mμ in a Beckman spectrophotometer, Model DU, primarily to detect the adenosine nucleotide peaks. Small aliquots of each fraction were plated on aluminum planchets, dried at 60—80° to remove ammonium formate, and the radioactivity was counted 30 seconds in a Tracerlab Sc-18 windowless flow counter. The procedure could readily detect the presence in one fraction of 0.5 % of the ^{14}C used in each experiment. The fractions containing each radioactive peak were later combined and the amounts of ^{14}C were determined again by plating and counting more accurately. The amount of ^{14}C in each peak recorded in Tables 1—4 is given as the percent of the total *summated* radioactivity in the entire chromatogram. The reproducibility of recovery of the radioactivity used in the experiment was within about ± 10 %.

The location of the various peaks found on these chromatograms was as follows: Uridine and uracil ("U") were in fraction 1 (containing the effluent from the sample placed onto the column) and fraction 2, AMP was in fractions 6—7, UMP in 18—20, orotic acid and ADP in 22—24, "UX" in 32—33, "UDP" and ATP in 35—37 and "UTP" in 42—43. When the columns were identical with regard to dimensions and flow rate the position of these peaks was reproducible. The "UX", "UDP" and "UTP" peaks are so designated because their chromatographic behaviour corresponded to that of the known compounds described in previous work [17], although in the present work they were not rigorously identified.

Enzyme fractionation. The fractionation procedure was used for rat, pigeon and chicken liver preparations. It will be described here for chicken liver.

The particle-free extract from 60—80 g of liver was fractionated by the addition of solid ammonium sulfate to obtain the protein precipitable between 35 % (0.245 g of ammonium sulfate per ml) and 45 % of saturation. This fraction contained most of the activity towards orotic acid. The precipitate was dissolved in 30 ml of 0.03 *M* phosphate buffer, pH 6.6, and dialyzed against 2 successive 2 liter volumes of the same buffer, at 1—2°, for a total of 2 hours. The protein solution was made to 50 ml with the buffer and 10 ml of methanol was added at the rate of 1 ml per minute while the temperature was being lowered to —5°. After another 10 minutes the precipitate was centrifuged at —5°. This precipitate contained little activity. A mixture of 5 ml of 0.08 *M* sodium acetate buffer, pH 4, and 1 ml of methanol was added slowly at —5°, followed by centrifugation as before. This precipitate contained most of the activity. The pH of the supernatant solution was 6.0. A third precipitate obtained by lowering the pH to 5.5 in a similar way was usually inactive. The active fraction, termed the "pH 6 methanol" fraction, was dissolved in 10 ml of 0.02 *M* phosphate buffer, pH 7.2, lyophilized and stored at 0°. This preparation retained its activity well for at least 2 weeks.

Direct spectrophotometric assay of enzyme activity. With the partially purified enzyme fractions a direct spectrophotometric assay for the disappearance of orotic acid was possible. A solution of 0.3 ml volume containing 0.1 μmole of non-radioactive orotic acid, 0.2 μmole of ATP, 0.2 μmole of ribose-5-phosphate, 1.25 μmole of $MgCl_2$, 15 μmole of phosphate buffer, pH 7.4, and 0.5—1.5 mg of lyophilized enzyme was incubated for periods up to 2 hours at 37°, then chilled during the addition of 1.25 ml of cold 0.5 *N* perchloric acid, and centrifuged. For each determination a second solution was prepared and treated identically with the first except that the orotic acid was omitted. The supernatant solutions, in 1 ml quartz cuvettes, were read at 260, 280, 300 and 350 mμ against an appropriate perchloric acid blank. To obtain the change in spectrum resulting from the action of the enzymes on the orotic acid, the readings of the "minus orotic" sample were subtracted from those of the "orotic" sample. This correction was necessary because during the incubation changes in the spectrum occurred, apparently arising from the protein. As a measure of the initial concentration of orotic acid, a solution of orotic acid in perchloric acid (without substrates or enzyme) was also prepared and analyzed in

an identical way. The amount of orotic acid converted was calculated directly from the decrease in the corrected reading at 300 mμ, where neither ATP nor UMP absorb. The readings at 260 and 280 were made to check on the formation of UMP, which absorbs maximally at 260 mμ. Small corrections for the light absorption of traces of colloidal protein were made by substracting the readings made at 350 mμ from the readings at 300 mμ.

Preparation of ribose triphosphate. A solution of 25 ml volume, containing 150 μmole of ribose-5-phosphate, 100 μmole of ATP, 100 μmole of $MgCl_2$, 950 μmole of phosphate buffer, pH 7.4, 900 μmole of NaF, and 200 μmole of reduced glutathione, was incubated at 37° with 20 mg of the "pH 6 methanol" enzyme. These conditions are basically those of Kornberg, *et al.*[11]. The reaction was followed by the use of adenylic acid deaminase to determine the amount of AMP produced from the ATP [18]. For this determination, 15 μl aliquots were removed into 1.0 ml of pH 6.0 citrate buffer and the change in light absorption at 265 mμ was determined upon the addition of the deaminase. When no further formation of AMP could be detected by this test (about 1.5 hours), the reaction was stopped by pouring the solution directly onto a 12 cm × 2 cm Dowex-1 (formate) column. The column was washed well with water and gradient elution was begun with water in the 500 ml mixing flask and with 2 *M* sodium formate of pH 5 in the reservoir flask. About 100 fractions of 6 ml volume were collected; these were analyzed for their ribose content and light absorption at 260 mμ. The ribose triphosphate was eluted in 5 fractions between the AMP and ADP. These fractions were low in light absorption. The combined fractions were adjusted to pH 7.0 with KOH, 0.5 ml of 2 *M* barium acetate was added, and the ribose triphosphate was precipitated by 1 volume of cold ethanol. After one hour at 0°, the precipitate was collected by centrifugation and washed successively with cold 50 % ethanol, ethanol and ether. Analysis of the best of these preparations, used for the work described later, indicated a ratio of ribose: acid-labile phosphate: total phosphate of 1 : 2.04 : 3.01 and a purity of 42 % (calculated with 2.5 Ba per ribose).

Ribose-1,5-diphosphate was prepared from ribose-1-phosphate and glucose diphosphate with crystalline mutase according to Klenow [19]. Purification was effected by gradient elution chromatography with 1 *M* sodium formate, pH 5, and precipitation with barium acetate as described for ribose triphosphate. The substance had a ratio of ribose: acid-labile phosphate: total phosphate of 1.0 : 0.99 : 2.06 and was 77 % pure, calculated as the dibarium salt.

Uridine-2-^{14}C and uracil-2-^{14}C were prepared from radioactive UMP by hydrolysis with 98 % formic acid at 175° for 4 hours followed by chromatography on a starch column [20].

RESULTS

The conversion of orotic acid to the uridine phosphates was initially tested in homogenates of rat liver metabolizing hexose diphosphate. The chromatographic analysis for the orotic acid, uridine nucleotides and adenosine nucleotides was employed. Small conversions of the orotic acid to uridine nucleotides and the "U" peak were noted when the homogenates were metabolizing the hexose diphosphate either aerobically or anaerobically. In both systems the amount of conversion appeared to be dependent upon the length of time the ATP levels were maintained. Both the maintenance of ATP and the conversion of orotic acid were then found to be greatly improved in the glycolyzing system when the particulate cell components were first removed by high speed centrifugation. Anaerobic incubation was not necessary in the particle-free system. The enzymes metabolizing orotic acid to the uridine nucleotides occur therefore in the non-particulate portion of the cytoplasm and, judging by the recovery of total activity, appear to be located primarily in this fraction.

A large number of exploratory experiments were carried out by incubating extracts of rat and pigeon liver with various combinations of substrates, activators and inhibitors. The relative amounts of orotic acid converted to

"U" and the various uridine nucleotides as well as the relative amounts of the adenosine nucleotides were determined chromatographically. When the particle-free extracts were dialyzed they were no longer active in the conversion unless hexose diphosphate, DPN and magnesium ion were added. (The addition of ATP was not necessary; however traces of ATP, perhaps arising from AMP in the DPN, were detectable chromatographically at the end of the incubations.) Phosphoglyceric acid was capable of partially replacing hexose diphosphate in this system. No conversion was obtained in the absence of either magnesium ion or DPN, nor in the presence of the glycolysis inhibitor iodoacetate *. It was apparent that the hexose diphosphate was serving both as a source of energy (*via* glycolysis and ATP) and of the ribose moiety of the uridine nucleotides.

The dialyzed extracts of pigeon liver (but not of rat liver *) could also be activated by addition of ribose-5-phosphate, provided ATP and magnesium ion were also present. DPN was not required. The full activity of the extract was not obtained in this way, apparently due to the destruction of ATP, since no ATP was detectable at the end of the incubation. However, the ATP level could be maintained and full activity obtained in both rat and pigeon liver extracts by the addition of phosphoglyceric acid, to serve as an energy source for the regeneration of ATP. (Phosphoglyceric acid in the absence of DPN could not replace ribose phosphate in this system.) From these data and from the work of Saffran and Scarano [10], it seemed probable that the ribose phosphate was providing the ribose moiety of the uridine nucleotides much more directly than did the hexose diphosphate and that ATP was a necessary reactant. Knowledge of the multiplicity of enzymes in the extract could not permit such an assumption to be conclusive, however **.

In the chromatograms of these reaction mixtures, five radioactive peaks in addition to the orotic acid were usually obtained. The first of these, the "U" peak, was in some experiments found to contain primarily uracil and some uridine as the radioactive components. For this work a warm butanol extract of the dried "U" peak (collected from a number of experiments) was chromatographed on a starch column with butanol-water [20]. The radioactive peaks were identified by their spectrum, R values and by recrystallization with authentic carrier uracil (solvent: water) and uridine (solvent: alcohol-ethylacetate).

The second peak contained UMP, which was identified by spectrum, phosphorus and ribose content, and by liberation of the phosphate group by snake venom 5′-nucleotidase [21, 22]. The three following peaks, labeled "UX", "UDP" and "UTP", corresponded in their chromatographic behaviour to the UDP-hexose, UDP and UTP of rat liver [17, 22], and contained uridine-5′-phosphate which could be liberated by hydrolysis with 1 N H_2SO_4 at 100° for 15 minutes. From larger scale incubation mixtures, the "UX" and "UDP" peaks were isolated by rechromatography with pH 5 ammonium formate [17].

* In pigeon liver extracts, which destroyed ATP much less rapidly than did rat liver extracts, some conversion of orotic acid could be obtained in the presence of iodoacetate by the addition of sufficient ATP (10—20 μmoles).

** Ribose phosphate and magnesium ion *in the presence of DPN* were also capable of generating ATP and effecting the conversion of orotic acid.

Analysis of both peaks indicated the ratio of uridine: total phosphate to be 1 : 2. The presence of either a hexose or a pentose in the "UX" peak could not be clearly determined*. Insufficient "UTP" was obtained for clear analyses. All of these peaks were usually found in the chromatograms of both the "hexose diphosphate" and "ribose phosphate" systems. The relative amounts of the peaks varied with the length of incubation, ATP level, age of the extract and the tissue source.

A number of tissues and one micro-organism were examined for their ability to carry out these conversions in the "ribose phosphate system". In these incubations, 3 mg of a muscle enzyme preparation [16] was added to assist maintenance of ATP; the muscle preparation itself had no activity towards orotic acid in the "ribose phosphate system". The results are presented in Table 1.

Table 1. Formation of uridine nucleotides from orotic acid-2-^{14}C by particle-free extracts of various tissues.

Tissue	Percent of total ^{14}C in chromatographic peaks					
	"U"	UMP	OR	"UX"	"UDP"	"UTP"
Pigeon Liver *	1	19	36	9	22	13
Chicken Liver	2	18	0	14	25	41
Rat Liver	11	52	0	27	9	1
Mouse Liver	24	53	0	20	3	0
Chicken Pancreas	0	0	0	4	2	94
Rat Kidney	19	7	68	5	1	0
Rat Spleen	41	47	0	6	6	0
Rat Heart	0	0	98	0	2	0
Mouse Kidney	14	27	53	5	1	0
Mouse Tumor **	4	23	25	16	15	17
E. coli ***	16	9	30	8	14	23

Each extract was prepared from 400 mg of tissue and incubated for 40 minutes at 37° with 0.5 μmole of orotic acid (500 000 c/min) in the "ribose phosphate system" (see text) with the addition of phosphoglyceric acid and muscle enzyme [16]. Cold perchloric acid extracts of the reaction mixture were made and chromatographed on Dowex-2 (formate) by elution with formic acid and ammonium formate. The radioactive peaks obtained are labeled "U" for uracil and uridine, UMP for uridine monophosphate, OR for remaining orotic acid, and "UX", "UDP" and "UTP" for the uridine pyrophosphate nucleotides.

* Phosphoglyceric acid and muscle enzyme *not* added.

** Spontaneous mammary tumor from C3H mice, kindly provided by Dr. Georg Klein.

*** Cell-free extract (prepared by repeated freezing and thawing from 50 mg of lyophilized *Escherichia coli*).

* The "UX" peak of an experiment with rat liver extract, ribose phosphate and phosphoglyceric acid was later rechromatographed and found to contain 43 % UDP-Glucose and 42 % UMP (the latter arising by degradation). For the spectrophotometric demonstration of the UDPG (by means of uridyl transferase, phosphoglucomutase and Zwischenferment [18]), we wish to thank Civ. Ing. Agnete Munch-Petersen.

A number of experiments were carried out with rat and pigeon liver in attempts to determine which of the radioactive products was formed first. Time curves indicated UMP to be the dominant peak at early time intervals (*cf.* Ref.[13], Fig. 1). The addition of non-labeled UMP to the incubation mixture (Table 2) increased the relative amount of incorporation into the UMP peak initially and greatly diminished the incorporation into the uridine pyrophosphates. This indicated the UMP to be the first uridine nucleotide product. The large shift of radioactivity into the "U" peak in the later time intervals of the experiment was interpreted to mean that the "U" peak was formed primarily by degradation of the UMP*. The addition of non-radioactive uridine did not greatly affect the distribution of radioactivity, which eliminated this compound from consideration as an intermediate in the conversion. Later work with partially purified enzymes verified these interpretations.

Table 2. Formation of uridine nucleotides from orotic acid-2-^{14}C: Effect of added pools of non-radioactive UMP or uridine.

Non-radioactive pool	Incubation Time Minutes	Percent of total ^{14}C in chromatographic peaks					
		"U"	UMP	OR	"UX"	"UDP"	"UTP"
UMP	5	0.3	2.4	96.0		0.8	0.5
UMP	10	1.7	5.7	89.0		1.9	1.7
UMP	40	30.5	10.4	55.4	1.0	2.7	
Uridine	40	1.7	0.4	63.0	12.7	9.5	12.7
None	40		1.0	59.0		40.0	

Particle-free extracts from 200 mg of rat liver were incubated at 37° with 0.5 μmole of orotic acid (500 000 c/min) in the "hexose diphosphate system" (see text). Pools of non-radioactive UMP (4 μmole) or uridine (2 μmole) were added as noted. The reaction mixtures were analyzed as in Table 1.

The enzymes of the particle-free extract were fractionated in order to determine which substrates and products were immediately concerned in the utilization of orotic acid. The fractionations were carried out with preparations from rat, pigeon and chicken livers. The latter proved to be the best source for the larger scale preparations, although similar results were obtained with all these tissues. The preparation of the active 35—45 % ammonium sulfate and the "pH 6 methanol" fractions is described earlier.

The 35—45 % ammonium sulfate fraction was tested in the "ribose phosphate system" (with the addition of the muscle enzyme preparation) and analyzed by chromatography. The results (Table 3) indicated that the enzymes

* That the "U" peak may be further metabolized is indicated by Fig. 1 [13], in which the "U" peak was formed initially and disappeared at later time intervals. This may represent conversion back to the uridine nucleotides or possibly complete degradation [24]. In other experiments with rat liver extracts plus phosphoglyceric acid and muscle enzyme, some formation of uridine nucleotides from uridine has been noted (unpublished).

degrading UMP to "U" were nearly eliminated and that the enzymes phosphorylating the UMP were diminished. Other experiments showed that the destruction of ATP by this preparation was not rapid.

Table 3. Formation of uridine nucleotides from orotic acid-2-^{14}C by 35—45 % Am_2SO_4 enzyme fraction from rat liver.

Incubation Time Minutes	Percent of total ^{14}C in chromatographic peaks					
	"U"	UMP	OR	"UX"	"UDP"	"UTP"
5		1.5	95.0	1.0	2.0	0.5
10		5.5	85.5	5.5	2.5	1.0
20	1.0	12.5	67.0	9.0	7.5	3.0
40	5.0	41.0	30.0	12.0	11.0	1.0

An amount of the ammonium sulfate fractionated enzyme containing 23 mg of protein was incubated in the "ribose phosphate system" with phosphoglyceric acid and muscle enzyme [16] (see text).

The "pH 6 methanol" enzyme preparation was tested with ribose phosphate, ATP and magnesium ion by both the chromatographic and spectrophotometric analyses. When relatively small amounts of ATP and large amounts of ribose phosphate were employed (Table 4) only UMP was formed from the orotic acid, indicating that the enzymes causing the degradation of the UMP were absent and that ATP-ase activity was negligible. Although the inclusion of phosphoglyceric acid and the muscle enzyme in the incubation mixture caused the formation of small amounts of the uridine pyrophosphates, the results indicated that the enzymes phosphorylating the UMP had been greatly reduced by the fractionation and were not essential for the reaction. Table 4 also shows that when either radioactive uridine or uracil replaced orotic acid in the incubation mixture there was no formation of uridine nucleotides. The UMP was thus demonstrated to be the first of the uridine phosphate compounds to be formed.

It appeared likely from the work of Kornberg *et al.*[11,12] that this enzyme preparation contained at least three enzymes concerned with the orotic acid utilization. In order to test for the reactions described by Kornberg *et al.*, ribose triphosphate was prepared using the "pH 6 methanol" fraction. The isolated ribose triphosphate (presumably 5-phosphoribosyl pyrophosphate) was found to be capable of replacing ribose monophosphate and ATP, when tested with the "pH 6 methanol" fraction in the direct spectrophotometric assay (Table 5). No activity was observed with the ribose triphosphate in the absence of magnesium ion. A sample of authentic ribose diphosphate, prepared according to Klenow [19] was found to be inactive with this preparation, even in the presence of ATP. Since no accumulation of orotic acid ribotide could be detected with any of the extracts or enzyme preparations employed it was not possible to make further tests for the mechanism described by Kornberg *et al.*

Table 4. Formation of uridine nucleotides from orotic acid-2-^{14}C by "pH 6 methanol" enzyme fraction. Test of activity with uracil and uridine.

Experiment	Radioactive Substrate	Time, Minutes	Percent of total ^{14}C in chromatographic peaks					
			"U"	UMP	OR	"UX"	"UDP"	'UTP"
I	Orotic acid	5		2	98			
I	»	10		6	94			
I	»	20		22	78			
I	»	40		32	68			
I	»	60		42	58			
I	Uridine	20	100					
I	Uracil	20	100					
II	Orotic acid	30		40	50		2	8

Experiment I: The incubation mixtures contained 5 μmole of ribose-5-phosphate, 0.6 μmole of ATP, 20 μmole of $MgCl_2$, 60 μmole of phosphate buffer, pH 7.4 and 8 mg of lyophilized enzyme (6.8 mg of protein). The radioactive substrates were 0.5 μmole of orotic acid (500 000 c/min) or 0.05 μmole of uridine (37 500 c/min) or 1 μmole of uracil (750 000 c/min). Total volumes were 2.5 ml.

Experiment II: The incubation mixture contained 3 μmole of ribose-5-phosphate, 3 μmole of ATP, 20 μmole of $MgCl_2$, 60 μmole of phosphate buffer, pH 7.4, 0.5 μmole of orotic acid (500 000 c/min) and 15 mg of lyophilized enzyme (10.5 mg of protein) in a total volume of 3.0 ml.

Different enzyme preparations were used in Experiments I and II. The incubation temperature was 37°.

Table 5. Conversion of orotic acid by "pH 6 methanol" enzyme fraction. Test of ribose phosphate, ribose diphosphate and ribose triphosphate in direct spectrophotometric assay.

Ribose compound	μmoles of orotic acid converted at given incubation times			
	15 min.	30 min.	45 min.	60 min.
RMP				0.000
RMP + ATP	0.010	0.021	0.032	0.039
RDP				0.000
RDP + ATP				0.000
RTP	0.012	0.023	0.028	0.031
RTP + ATP				0.036

1.0 mg of lyophilized enzyme (containing 0.8 mg of protein) was used for each determination in the direct spectrophotometric assay (see text). The initial amounts of the reactants in the assay were: 0.10 μmole of non-radiactive orotic acid, 0.20 μmole of ribose-5-phosphate (RMP), 0.15 μmole of ribose-1,5-diphosphate (RDP), 0.12 μmole of ribose triphosphate (RTP), and 0.20 μmole of ATP.

DISCUSSION

The objective of this work has been to examine some of the enzymatic steps in the utilization of orotic acid for synthesis of the polynucleotides in living tissues. It has been established that the initial reaction in the utilization is the condensation of orotic acid and ribose triphosphate with the subsequent formation of UMP. The enzymes of the non-particulate portion of the cytoplasm were responsible for these reactions. (It was, however, not determined whether such enzymes are *absent* in the cell particles.) Other enzymes of this cytoplasmic fraction were found to be capable of splitting the UMP to produce uracil and uridine, as well as of phosphorylating it to form several uridine pyrophosphate nucleotides. A different cell fraction, the mitochondrial fraction of the cytoplasm, appears to be responsible for the reactions leading to the *formation* of orotic acid [5].

Kornberg *et al.*[11, 12], have described the intermediate steps in the conversion of orotic acid to UMP. An enzyme prepared from pigeon liver reversibly catalyzed the reaction of ATP with ribose-5-phosphate to form 5-phosphoribosyl pyrophosphate. Enzymes from both pigeon liver and yeast caused coupling of the latter compound with orotic acid by elimination of pyrophosphate, followed by decarboxylation of the product to form UMP. The reversible formation of orotic acid ribotide as an intermediate was demonstrated with a yeast preparation freed of orotic acid ribotide decarboxylase activity. The decarboxylation step appeared to be essentially irreversible. We have confirmed the initial coupling of orotic acid with ribose triphosphate and, although our enzyme preparation was not free of decarboxylase activity so that the formation of orotic acid ribotide was not demonstrated, it seems probable that our system followed the reactions described by Kornberg *et al.*

A number of tissues have been examined for their ability to convert orotic acid to uridine nucleotides. Of these, liver, pancreas and spleen were relatively active, tumor and kidney were less active and heart muscle was nearly inactive. The amount of activity toward orotic acid appears to correlate roughly with the content and "turnover" of the ribonucleic acid in the tissues examined. The differences in the relative amounts of the uracil compounds formed by these tissues probably reflects different balances among the various enzyme activities involved. Such differences may be of value in preparative work; the high yield of "UTP" with the chicken pancreas is especially noteworthy in this respect. In previous work [2] a rapidly growing Flexner-Jobling tumor and other extrahepatic tissues of the *living* rat utilized only small amounts of the injected orotic acid for nucleic acid synthesis, in apparent discrepancy with work on other precursors in *in vitro* systems. The present report supports the suggestion that many tissues (including tumors) have the potential ability to utilize orotic acid, but that factors such as circulation and absorption greatly affected the results obtained *in vivo*. The conversion of orotic acid to uridine nucleotides has also been found to occur in *E. coli* (present report), yeast [11, 12], *S. aureus* [23] and mouse ascites tumor *.

* The conversion of orotic acid-4-^{14}C to the free uridine and cytidine nucleotides has also been found in ascites tumor by Dr. Hanns Schmitz (personal communication) and in oxidizing fortified homogenates of rat liver by Liselotte I. Hecht (presented at the Meeting of the American Association for Cancer Research in April, 1954).

With regard to the activity of the enzyme system converting orotic acid to UMP, the extract from 1 gram of liver could convert 1.25 μmole of orotic acid in 30—40 minutes. Although this activity does not appear great, it may be calculated that the total uridine nucleotides of the liver (about 1 μmole per gram of fresh weight) could thus be renewed from orotic acid in about one-half hour. This rate does not seem to be grossly inadequate for the supply of the uracil moiety entering the nucleic acids. (The total ribonucleic acid contains roughly 3 μmole of uracil per gram fresh weight of liver and the renewal time of the polynucleotides is of the order of many hours [2]). Whether the rate of *formation* of orotic acid in the liver is adequate to account for the uridine nucleotides formed remains to be determined. Reichard [5] has shown the rate of synthesis of ureidosuccinic acid, a precursor of orotic acid, to be more than adequate. Other pathways of uridine nucleotide formation may well exist; in fact work with other organisms indicates that uracil and possibly uridine may also be utilized to varying extents [23-26] for synthesis of polynucleotides. Whether or not this utilization proceeds through the uridine nucleotides has not been established.

The cytosine of the polynucleotides becomes labeled when orotic acid is used as precursor in living rat [1,2]. Under these conditions the free cytidine nucleotides of the liver also become labeled to a specific activity one-fourth to one-half that of the uridine nucleotides *. In the present work with the particle-free extracts, no significant formation of radioactive cytidine phosphate was noted. This negative finding may indicate either that the system used was inadequate with regard to substrates or that some of the cell particulates are involved. Further investigation of the formation of cytidine phosphate is in progress.

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Some Copolymers of Vinyl Alcohol-Acrylonitrile

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Copolymers of vinyl alcohol and acrylonitrile were prepared by selective hydrolysis of emulsions of the corresponding vinyl acetate-acrylonitrile compounds with ammonia at slightly elevated temperature. The presence of acrylonitrile groups in the polyvinyl alcohol chains does not lead to increased molar cohesion and a corresponding increase in filogenic properties. However, the polymers are compatible with pure polyacrylonitrile in solution (solvent: dimethyl formamide) and synthetic fibres can be prepared from such binary systems. Compared with pure polyacrylonitrile fibres the most obvious changes are the enhanced affinity for dispersed (acetate) and acid dyestuffs. The observed decrease in tenacity and increase in elongation at break is expected.

It seems hard to understand why polyvinyl acetate has never been obtained in a crystalline state when it is remembered that the polyvinyl alcohol, obtained by alkaline hydrolysis of the acetate, is often easily crystallizable. The degree of regularity in structure is almost identical in both polymers, provided that saponification is complete. The head-to-tail arrangement in the structures dominates, as is well established by degradation studies [1]. One interpretation of the X-ray diffraction pattern of polyvinyl alcohol [2] even indicates that all of the hydroxyl groups are placed on the same side of the polymer chain. (In the light of recent investigations [3] this seems far from being a general rule. The alternating distribution of the hydroxyl groups seems to be most common.) A *branching* effect is unlikely to be responsible for the poor crystallizability. It is not more probable for vinyl acetate than for other vinyl monomers — in any case it should have been indicated by a change in the physical behavior in solution. The solubility parameters E/V (cohesive energy density) and δ ($= \sqrt{E/V}$, corrected for dipole interaction related to polarizability of solvent) for polyvinyl acetate have been recently computed from molar attraction constants [4] and found to be close to those for polystyrene or polymethyl methacrylate. This implies that special deviations from the behavior of ordinary straight polymers are not to be expected in

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solvents showing a moderate or high cohesive energy density, such as acetone (c.e.d. 4 cal.ml^{-1} at 20° C) or dimethyl formamide (c.e.d. about 12 cal.ml^{-1} at 20° C).

Additional studies on the crystallizability of certain types of polyvinyl alcohol copolymers [5] are very illuminating. Generally speaking crystallization may occur, even if the second mer component is distributed at random along the head chain, provided that the side groups can be "contained" in the space unit cell of polyvinyl alcohol. This is possible with mer units such as ethylene and tetrafluorethylene, but surely not in the case of vinyl acetate.

Thus it seemed interesting to find out something about the crystallizability of the copolymers of vinyl alcohol and *acrylonitrile*. Such a copolymer is obtained by selective hydrolysis of the corresponding vinyl acetate-acrylonitrile compound. The small difference in the space occupations of hydroxyl and nitrile groups permits a cautious supposition that the conditions for crystallizability might be fulfilled in this case, at least at some proper balance between the mer units of the polymer in a highly oriented state, such as a wet spun fibre. The main point of uncertainty is whether this class of copolymers retains the fibre-forming properties found in polyvinyl alcohol and polyacrylonitrile. Several factors of steric and energetic origin influence the softening point, melting point and second order transition temperature of the finished copolymer. This needs further discussion.

As is known from earlier investigations [6] the introduction of nitrile groups leads to increased density and a gradual increase in the intermolecular forces. This effect is, to a certain extent, counterbalanced by a melting point depression originating from the entropy changes caused by the introduction of a new structural element in the polymer chains. According to Flory [7,8] the melting point depression T_o—T is given by the formula

$$1/T - 1/T_o = -R/\Delta H \cdot \ln N_x$$

where R = the gas constant (in cals); ΔH = molar heat of fusion of the repeating head unit; N_x = mole fraction of the crystallizing constituent.

The formula applies to random copolymers and corresponds exactly to the classical expressions for freezing point depression and solubility-temperature gradients in dilute solutions of small molecules. For higher fractions of the new mer unit the cited formula evidently represents an oversimplification, but indicates that the melting point depression is mostly dependent upon the molar heat of fusion of the main repeating unit. It can be generalized by taking into account the interaction parameter between solvent and solute [9]. The molar heat of fusion of vinyl alcohol is not known — the simplest assumption is that it approximates the value for acrylonitrile. In the case of straight acrylonitrile polymer the large melting range (softening and decomposition) [8] indicates a high molar heat of fusion and a low entropy of fusion — the ratio between these magnitudes represents the hypothetical melting point T_o of the crystalline polymer. A high heat of fusion means a low melting point depression on the introduction of a second component. From thermodynamic viewpoint this only indicates limited flexibility of the molecular chains in the liquid state, possibly originating from repulsion between identical groups *along* the same chain [8]. But conditions are obviously different in a polymer where two mer

units are randomly distributed. The repulsion effect must be changed in some way, usually giving a more flexible molecule, capable of curling in the melt [10]. In this way the entropy change on melting is considerably increased and, for a given heat of fusion, the melting point is lowered in relation to the entropy gain. It seems probable already from these considerations that the second order transition range of the vinyl alcohol-acrylonitrile polymers is far below 100° C, because this magnitude generally follows the first order transition according to the Beamans rule [11], $T_{\text{melting}}/T_{\text{sec. order trans.}} = 1.5$.

EXPERIMENTAL

Preparation of the copolymers. The copolymers of acrylonitrile and vinyl acetate are easily prepared in emulsion systems. The dispersions show very good heat and frictional stability, unlike the corresponding polyacrylonitriles where stable emulsions are rare. It is apparent that the dispersed state offers the most convenient medium for the selective hydrolysis of the acetyl groups. In fact preliminary tests showed that a careful hydrolysis (pH 10, temp. 65° C, time 120 min) in the presence of (excess) ammonia did not attack the nitrile groups, as a blank of pure polyacrylonitrile immediately showed, but gave an acetyl content of the saponified copolymer usually below 0.75 %.

Table 1. Copolymerization of acrylonitrile-vinyl acetate.

Apparatus: Three-necked flask, volume 1 000 ml, equipped with thermometer, agitator and reflux condensor. Rate of stirring 300—400 r.p.m.

Composition of reaction mixture: Water 1 500 g; Activator: potassium persulphate 6 g; Emulsifier: Sodium lauryl sulphate 8 g; Amount of monomers 240 g; Ratio of monomers: See below; Time of reaction 120 min; Temp. of reaction 65—70° C.

Note. The reaction mixture was flushed with nitrogen before initiation of polymerization. The monomers were mixed and dispersed in the aqueous phase at room temperature before adding activator. After reaction, excess of monomer was removed at 65° C in vacuum using a slow current of nitrogen through the emulsion.

Prep. No.	1	2	3	4
Vinyl acetate, g	40	75	120	180
Acrylonitrile, g	200	165	120	60
mole ratio vinyl acetate/acrylonitrile	0.12	0.29	0.62	1.85
acetyl content of saponified polymer, %	0.81	0.44	1.19	1.01
Solubility of saponified polymer in				
a. Dimethylformamide	Clearly soluble, 20 % solutions are easily prepared			
2. Acetone	Insol.	Insol.	Sol.	Sol.
Av. visc. molecular weight in dimethylformamide *	78 000	57 000	42 000	37 000

* Calculated from a speculated K_m-value of 1.5×10^{-4}, and approximating the shape exponent in $[\eta] = K \cdot M^a$ to unity.

Remarks. The polymers obtained are all somewhat sticky when heated above 50° C. It is therefore necessary to carry out the precipitation of the hydrolyzed polymer emulsion at a low temperature — preferably below room temperature. If this direction is observed the polymer is obtained as a finely divided powder — similar in appearance (and colour) to sawdust.

VISCO-ELASTIC BEHAVIOR OF THE SAPONIFIED POLYMERS

After drying at 50° C, the polymers were dissolved in dimethylformamide at room temperature. Solutions containing 10 % polymer were easily filtered through sheets of cotton linters. The de-airated dopes were spread on glass plates and the solvent removed by drying at 100° C. The films were cut into strips and oriented by stretching over a hot surface. Some observations should be mentioned here:

1. At higher acrylonitrile contents (prep. 1—2) plastic flow on stretching occurs above 50° C, the oriented films being split into fibrilla in the flow direction by a high instantaneous load. The creep recovery is low after the stretching operation. The elongation on rupture at 20° C is about 20 %. A stretch ratio of 1 : 4 is obtainable in both specimens.

2. At lower contents of acrylonitrile (prep. 3 and 4) a creep recovery on stretching was detectable. Oriented films are not split into fibrilla by a sudden heavy load, on the contrary a pronounced rubbery character is apparent. Only a low resistance to plastic flow can be noticed above 50° C, indicating a *small* increase in orientation. No critical "draw limit" can be found and the typical "necking" effect on drawing has disappeared.

These observations indicate that the introduction of acrylonitrile groups into polyvinyl alcohol do not increase the fibre-forming properties. At higher contents of acrylonitrile the characteristics of acrylic fibres make their appearance, which is trivial in our present case.

Some indirect information, however, can be obtained from another aspect. It was observed that solutions of the "hydrolysed" copolymers in dimethylformamide are miscible in all proportions with solutions of pure polyacrylonitrile in the same solvent. This is to be expected, even if mutual miscibility of polymers is by no means an ordinary phenomenon. In this way a two-component fibre can be prepared where the influence of the copolymer, acting as a diluent, can be identified by comparison with the single-component fibrer, prepared under identical spinning conditions. Such a test was carried out with copolymer 4, and a pure acrylonitrile polymer, in accordance with the performance described below.

It is apparent from table 2 that the copolymer mainly acts as a "diluent" for the acrylic polymer, which is reflected in the decrease in tenacity and the increase in ultimate elongation. This might partly be due to an additive effect, derived from the fact that the copolymer units interfere with the structural elements of the polyacrylonitrile, thus both preventing and retarding orientation and final crystallization to a smaller extent. In any case both effects should co-operate in changing the structural elements in the Maxwell model of the two-component fibre which corresponds with the measurements above. From this point of view one is not allowed to negate the

Table 2. Wet spinning of a two-component fibre of polyacrylonitrile and a vinyl alcohol-acrylonitrile copolymer.

Blank (Sol. I): Solution of polyacrylonitrile (av. visc. M. W. = 70 × 10^3) in dimethylformamide, 15 % by weight.
(Sol. II) Spinning dope: One part by weight of copolymer 4 dissolved in dimethylformamide (15 %), mixed with three parts of Solution I.
Coagulating agent: Odourless Kerosene. Temp. of coag. agent: 135° C. Type of spinnerette: Nickel, 100 holes. Diam. of holes: 0.15 mm. Stretch ratio: 8. Rate of extrusion: 80 metres min^{-1}. Washing of the fibres: Boiling water. Drying: Room temperature.
Properties of the fibres obtained:

Type of fibre	Acrylic polymer	Acrylic polymer + copolymer
Denier	122	158
Tenacity, g. $denier^{-1}$	3.4	3.0
Elongation at break, %	17.1	23.6
Dyeability with dispersed (acetate) dyestuffs at 95° C*	1	3
Dyeability with acid dyestuffs at 95° C	0	2

* Figures in a relative scale:
0 = no visible sorption.
1 = visible.
2 = moderate.
3 = good.

contribution of the copolymer to the mechanical strength. The decrease in crystallizability of the main component might alone be sufficient to account for the main part of the decrease in tenacity in the two-component fibre. The increase in elasticity is a necessary consequence from high polymer thermodynamics. The enhanced affinity of the modified fibre for dispersed dyestuffs is also in agreement with the changes mentioned above.

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The Conjugation of Glycine with Cholic Acid and Benzoic Acid in Rat Liver Homogenate. Bile Acids and Steroids 21

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The formation of glycocholic and taurocholic acid has been shown to take place in a system containing microsomes and supernatant from rat liver, whereas the hippuric acid formation also requires the presence of mitochondria.

The taurine present in the rat liver has been shown to be quantitatively conjugated with cholic acid in spite of the presence of considerably greater amounts of glycine.

We have recently shown that both the "microsome fraction" and the "supernatant fraction" of rat liver homogenate are essential for the conjugation of cholic acid with taurine[1,2]. We have now been able to separate the system responsible for the taurocholic and glycocholic acid formation from that forming hippuric acid.

Taurocholic acid was formed in preference to glycocholic acid when both taurine and glycine were present.

EXPERIMENTAL

Liver homogenate of male rats (weight 200—250 g) was separated into "mitochondria fraction", "microsome fraction" and "supernatant fraction" as described elsewhere [2]. The homogenate fractions tested for enzyme activity were incubated with cholic acid, benzoic acid, ^{14}C-labeled glycine and ^{35}S-labeled taurine as shown in Fig. 1. The incubates were then extracted with 1/3 volume *n*-butanol [1], but the contents of the vessels had to be acidified to pH 1 to make also the hippuric acid extractable. This procedure has been shown to leave the free taurine in the water phase whereas taurocholic acid is extracted by the butanol [1]. Fig. 1 shows that also the glycine stays almost quantitatively in the water phase whereas the glycocholic and hippuric acids are extracted.

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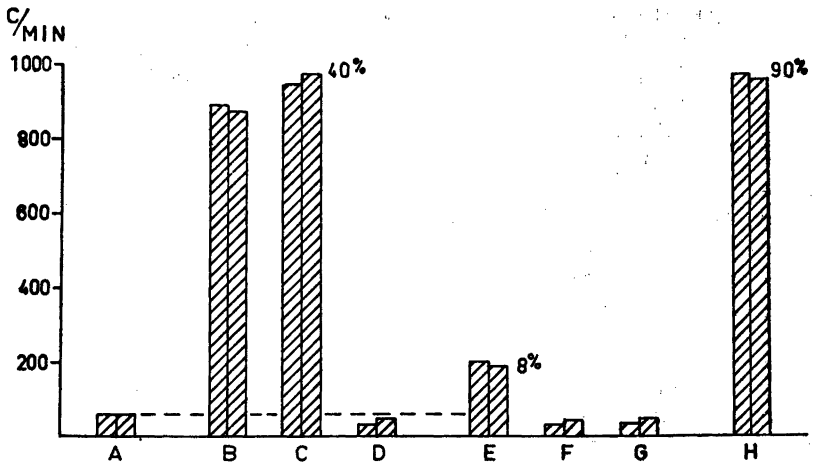

Fig. 1. Total volume of the vessels 1.75 ml. 1 ml 20 % homogenate added to each vessel. Concentrations: Potassium phosphate buffer pH 7.7 0.037 M, *sucrose 0.080* M, *nicotinamide 0.017* M, *magnesium chloride 0.00065* M. *ATP 1.2 μmole/vessel, versene 0.5 mg/vessel.*

A. 1 ml homogenate centrifuged at 400 g for 5 min. 1.8 μmole glycine-^{14}C representing 15 000 c/min/vessel.
B. As A + 2.5 μmole cholic acid.
C. 1 ml homogenate centrifuged at 12 000 g for 10 min. 1.8 μmole glycine-^{14}C + 2.5 μmole cholic acid.
D. 1 ml homogenate centrifuged at 100 000 g for 30 min. 1.8 μmole glycine-^{14}C + 2.5 μmole cholic acid.
E. As A + 2.5 μmole benzoic acid.
F. As C, but with benzoic acid instead of cholic acid.
G. As D, but with benzoic acid instead of cholic acid.
H. 1 ml homogenate centrifuged at 12 000 g for 10 min. 0.4 μmole taurine-^{35}S representing 7 250 c/min/vessel. + 2.5 μmole cholic acid.

Each vessel acidified with 0.1 ml conc. HCl and extracted with 0.62 ml of butanol. 0.1 ml butanol plated and counted. The double columns represent parallels.

The distribution of hippuric acid between the butanol/*N* hydrochloric acid phases in the concentration range used was estimated spectrophotometrically at 2 700 Å. The partition coefficient butanol/*N* hydrochloric acid was found to be 5.2. After equilibration of the acidified reaction mixture the volume of the aqueous phase was about 1.94 ml and that of the butanol phase 0.54 ml.

The distribution of glycocholic acid between the butanol/*N* hydrochloric acid phase was assumed to be the same as for taurocholic acid [1].

The amounts of the conjugates extracted with butanol was determined by measuring the radioactivity of aliquots plated directly under standard conditions [1].

RESULTS AND DISCUSSION

The two separate experiments done gave similar results. One of the experiments is shown in Fig. 1.

The liver homogenate used in this experiment was nearly five times as efficient in conjugating cholic acid with glycine (Fig. 1 B) as in conjugating

benzoic acid with glycine (Fig. 1 E). The 1.8 μmole glycine-^{14}C added to the systems gave under identical conditions rise to 0.72 μmole glycocholic acid and 0.15 μmole hippuric acid, respectively. Since some free glycine was presumably present in the liver homogenate, these amounts must represent the minimal amounts of newly formed conjugated acids. A content of 0.2 mg of free glycine per g rat liver has been reported by Wu[8].

Furthermore, there is a distinct difference in the localization of rat liver systems responsible for the formation of the glycocholic and hippuric acids. The "microsome" plus "supernatant" fractions retained in full the activity to synthesize glycocholic acid (Fig. 1 C), but were unable to bring about the formation of hippuric acid (Fig. 1 F). The finding that the mitochondria are essential for the hippuric acid synthesis is in accordance with the results of Kielly and Schneider[3] in mouse liver homogenate.

Both the "microsome" fraction and the "supernatant" are essential for the formation of glycocholic acid (Fig. 1 C, D). This localization is similar to that of the system forming taurocholic acid[2].

In order to compare the conjugation of cholic acid with taurine and with glycine, the concentration of preformed taurine in the homogenate had to be determined. Two vessels with homogenate from the same liver were incubated with 0.4 μmole taurine labeled with ^{35}S (Fig. 1 H). The activity recovered as taurocholic acid correspond to 90 % of the amount added. Spectrophotometric determination according to Sjövall [4,5,1] showed, however, that a total amount of 0.70 μmole taurocholic acid had been formed, *i.e.*, the total amount of taurine present at the start of the experiment must have been about 0.78 μmole, of which only 0.4 μmole was the labeled material added.

Similarly spectrophotometric determination of the formation of taurocholic acid when the system in addition to cholic acid and preformed taurine also contained ^{14}C-labeled glycine (1.8 μmole) (Fig. 1 C) showed the taurine to be almost quantitatively conjugated in spite of the glycine present. This finding agrees with the earlier observations of Bergström and Gloor[6] in human liver homogenates.

The enzymic systems conjugating cholic acid with taurine and glycine in the rat must therefore have greater preference for taurine; the cited results give a good explanation of the fact that rat bile contains only 5—10 % of the cholic acid conjugates in the form of glycocholic acid[7] in spite of the relatively high glycine content in rat liver. The glycine content of rat liver has been reported to be three times that of taurine on a molar base[8].

Whether the conjugation of cholic acid with glycine and taurine is performed by the same enzyme system is as yet an open question.

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On the Ferric-ion Catalyzed Decomposition of Hydrogen-Peroxide

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It is agreed that a reaction pattern proposed by J. Koefoed, which implies the closed sequence

$$Fe^{++} + FeO_2H^{+2} \rightleftharpoons Fe_2O_2H^{+4} \quad 12$$
$$Fe_2O_2H^{+4} \rightleftharpoons Fe^{+3} + FeO_2H \quad 23$$
$$FeO_2H^{+} + H_2O_2 \rightarrow O_2 + H_2O + HO + HO^{-} + Fe^{+2} \quad 31$$

and which can be made to agree with Sten Andersen's experiments is in better agreement with general experimental evidence than an open sequence formerly proposed by Sten Andersen and the present author. On the basis of tracer experiments from elsewhere it is concluded that the intermediate FeO_2H^+ is a ferrous perhydroxo complex and not, as advocated by J. Koefoed, an oxo-hydroxo derivative of Fe(IV).

Recently J. Koefoed [1] proposed a reaction pattern for the ferric-catalyzed decomposition of hydrogen-peroxide which
1) agrees with Sten Andersen's chronometric integral [2] $t = A_1 \ln a/x + A_2 (1/x - 1/a)$ (x concentration of hydrogen-peroxide at time t, a the same at $t = 0$),
2) has the property of containing a closed sequence (a chain mechanism) which the pattern proposed by Sten Andersen and the present author has not, and finally 3) operates with ferrous ion as an intermediate which the one mentioned above does not.

As it is an experimental fact that the decomposition in question is strongly retarded by small amounts of certain substances and that during the decomposition ferrous ions can be analytically [3] detected it is obvious that Koefoed's pattern is in better general agreement with the experimental facts taken at their face-value than the one mentioned above.

There is, however, one detail in Koefoed's pattern which may need a little discussion and another which seems to be in disagreement with well established experimental findings. We shall begin with a discussion of the latter.

Koefoed admits the well known fact that kinetics can tell nothing about the constitution of the intermediates.

He tries, however, by using arguments from elsewhere to make it probable that the intermediate of the composition FeO_2H^+ is not a ferrous-perhydroxo complex but an oxo-hydroxo compound of Fe(IV), *viz.* $O : \overset{+}{F}eOH$. His arguments can best be discussed by means of his closed sequence which is

$$Fe^{+2} + FeO_2H^{+2} \rightleftharpoons Fe_2O_2H^{+4} \qquad 12$$

$$Fe_2O_2H^{+4} \rightleftharpoons FeO_2H^+ + Fe^{+3} \qquad 23$$

$$FeO_2H^+ + H_2O_2 \rightarrow O_2 + H_2O + HO^- + Fe^{+2} \qquad 31$$

This is so constructed that it makes it possible to remain in agreement with all Sten Andersen's results on the ferric ion catalyzed reaction.

It is a fact that during the overall reaction a pair of antiparallel electronic spins are converted into a pair of parallel spins and Koefoed now argues that this conversion takes place during the step 23, which would support the assumption of the constitution $O\overset{+}{F}eOH$ for the intermediate. His argument seems, however, to be highly artificial: There is complete agreement in views on the constitution of FeO_2H^{+2} as a perhydroxo analogue to $FeOH^{+2}$ and on the constitution of $Fe_2O_2H^{+4}$ as the perhydroxo analogue to a ferrous-ferric hydroxo complex of well known type. Why not therefore assume that the ferrous and the ferric ions in 12 and 23 simply keep their spins and assume, as we then must, that the "spin-conversion" takes place in the irreversible reaction 31? One might even say that such an assumption is probable in view of the strong tendency for ferrous ions to form coordinated complexes with certain anions. This would lead in our case to an accumulation of two perhydroxo ligands around the central ion, the resulting complex being so to speak explosive. Anyhow there is direct evidence in favour of the view that FeO_2H^+ is actually a ferrous-perhydroxo ion and not an oxo-hydroxo derivative of Fe(IV). Two groups of authors, Baertschi [4] on one side and Cahill and Taube [5] on the other, agree in stating, on the basis of numerous tracer experiments, that in the reactions of hydrogen-peroxide the evolved oxygen is derived "cleanly from the hydrogen-peroxide" (Cahill and Taubes expression) that is, there is no measurable reversible exchange of oxygen between the perhydroxide and the solvent water. This is true in all the cases investigated and particularly in our case which has actually been investigated by Cahill and Taube. Now from general knowledge and from experiments it appears that oxygen in metal-hydroxo compounds is readily exchangeable with oxygen in the solvent water. It would therefore be a lengthy and, as the present author believes, an impossible task to prove that the assumption of FeO_2H^+ being an oxo-hydroxo compound does not contradict the tracer experiments.

Koefoed has, however, another argument which according to him should make the assumption, that FeO_2H^+ be a perhydroxo compound, improbable: Let us add to the equations 12 and 23 the equation for the reaction

$$HO^-_2 + Fe^{+3} = FeO_2H^{+2}$$

We then get as the resultant of the three processes

$$Fe^{+2} + HO^-_2 = FeO_2H^+$$

in other words: ferric ions should catalyze the latter reaction. (Koefoed prefers to add the equilibrium

$$H_2O_2 + Fe^{+3} = FeO_2H^{+2} + H^+$$

with a corresponding change in the resultant equation, but the difference is so far as we can see to all intents and purposes immaterial.) He now says that it is not very likely that the reaction in question "should be much promoted by this catalyst", in other words the direct formation of FeO_2H^+ from Fe^{+2} and HO^-_2 (the bye-pass) should be faster than that through the reactions 12 and 23. However, as shown in the appendix the problem in question cannot be decided that way. A rational treatment shows 1) that only the kinetic experiments themselves can decide whether the existence of the bye-pass is of significance or not, 2) that Sten Andersen's experiments indicate that its existence is insignificant in the range of ferric ion concentrations investigated and 3) that this result would be *a priori* probable rather than improbable.

We see therefore no reason to admit other assumptions than that the intermediate FeO_2H^+ simply is a ferrous perhydroxo compound in which way any contradiction with the tracer-experiments automatically disappears.

We now pass on to the minor first point. As initiator Koefoed assumes the net reaction $FeO_2H^{+2} + Fe^{+3} + 2\ H_2O \rightarrow 2\ FeO_2H^+ + 3\ H^+$ divided into two steps as follows

$$H_2O + FeO_2H^{+2} \rightarrow FeO_2H + HO + H^+$$
$$HO + Fe^{+3} + H_2O \rightarrow FeO_2H^+ + 2H^+$$

Actually, in agreement with Koefoed's statement, only the left hand side of the first partial reaction is known from the kinetics, water of cause being kinetically unimportant. Therefore if we don't like to state more than we know the equation ought to be written

$$H_2O + FeO_2H^{+2} \rightarrow Y \qquad 0y$$
$$Y + Fe^{+3} + H_2O \rightarrow 2FeO_2H^+ + 3H^+ \qquad y3$$

Anyhow it seems certain that in Koefoed's initiating reaction part of the perhydroxide must come from the solvent water. This will however probably not contradict the tracer experiments because of his basic and most probably correct assumption of a large chain length. Even a low value of say 100 would probably prevent its detection if only the chain-mechanism itself does not give rise to exchange of oxygen with the solvent water. We have therefore no objection in principle to Koefoed's initiating sequence.

The present author has also something to say about Koefoed's treatment of Sten Andersen's experimental results concerning the cupric-ferric ion catalyzed reaction. But this discussion can only be completed in cooperation with Sten Andersen, a cooperation which because of Sten Andersen's temporary absence must be postponed to a later date. Be it enough to state here that it may (not must) be true that Sten Andersen's experiments admit mechanisms (reaction patterns) which do not lead strictly to his chronometric integral of the type $t = A_1\ln(a/x) + A_2(1/x - 1/a)$, which the one proposed by Koefoed admittedly does not.

It must be added, however, that a reaction-pattern including Koefoed's chain-mechanism has been constructed which agrees strictly with Sten Andersen's chronometric integral, and with the aid of which his numerical values for A_1 and A_2 can be reproduced with some accuracy.

APPENDIX

Let us assume, besides the reaction

$$Fe^{+2} + FeO_2H^{+2} \rightleftharpoons Fe_2O_2H^{+4} \qquad 12$$
$$Fe_2O_2H^{+4} \rightleftharpoons Fe^{+3} + FeO_2H^{+} \qquad 23$$

also the bye-pass

$$Fe^{+2} + HO^{-}_2 \rightleftharpoons FeO_2H^{+}$$

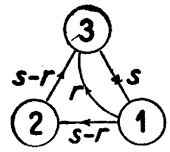

Fig. 1. Flow sheet for the closed sequence 12, 23, 31 with bye-pass 13. The steady state currents are as shown by the letters and arrows.

The flow-sheet of the reaction is shown in Fig. 1, where the currents corresponding to steady state have been indicated. Denoting the probabilities of the reaction of the intermediates Fe^{+2} and FeO_2H^{+} according to the bye-pass reaction by u_{13} and u_{31} respectively we get

$$x_1 = r/u_{13} + su_{31}/u_{13}w_{31}$$
$$x_1 = (s—r)(1 + w_{21}/w_{23})/w_{12} + sw_{21}w_{32}/w_{12}w_{23}w_{31}$$

From the principle of microscopic reversibility we have the equation

$$w_{12}w_{23}u_{31} = w_{21}w_{32}u_{13}$$

which means that, with $w_{21}/w_{23} \gg 1$

$$sw_{21}/w_{12}w_{23} = r(1/u_{13} + w_{21}/w_{12}/w_{12}w_{23})$$
$$r/s = 1/(w_{12}w_{23}/w_{21}u_{13} + 1) = 1/(w_{32}/u_{31} + 1)$$
$$= u_{31}/(w_{32} + u_{31})$$

This yields

$$\frac{x_1}{s} = \frac{u_{31}}{u_{13}}\left(\frac{1}{w_{32}+u_{31}} + \frac{1}{w_{31}}\right)$$

For x_2/s we similarly get

$$\frac{x_2}{s} = \left(1—\frac{r}{s}\right)/w_{23} + w_{32}/w_{23}w_{31}$$
$$= \frac{w_{32}}{w_{23}}\left(\frac{1}{w_{32}+u_{31}} + \frac{1}{w_{31}}\right)$$

Replacing u_{31}/u_{13} by $w_{32}w_{21}/w_{12}w_{23}$
we finally get

$$\frac{x_1x_2}{s^2} = \frac{w_{21}}{w_{12}}\left(\frac{w_{32}}{w_{23}}\right)^2\left(\frac{1}{w_{32}+u_{31}} + \frac{1}{w_{31}}\right)^2$$

which is identical with the corresponding expression obtained without the bye-pass if $u_{31} \ll w_{32}$.

Now u_{31} is the probability in unit time that FeO_2H^+ shall dissociate into Fe^{++} and HO^-_2 and w_{32} the probability that it shall unite with a ferric ion to form $Fe_2O_2H^{+4}$. It would seem *a priori* rather probable that the former probability is much less than the latter at reasonable concentrations of ferric ions. As, however, w_{32} must be of the form k_{32} $[Fe^{+3}]$ it might be possible by making experiments at very low ferric ion concentrations to detect the influence of the bye-pass, but on account of the low concentration of the catalyst such experiments would last an exceedingly long time. Anyhow, only the kinetic experiments themselves can decide with reasonable certainty on the ratio u_{31}/w_{32} and the actual result derived from Sten Andersen's experiments, that $u_{31}/w_{32} \ll 1$, is not at all improbable *a priori*, or rather, it seems to be probable and quite natural.

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Utilization of Ion Exchangers in Analytical Chemistry

XXIX. Sorption and Elution of Some Low-Molecular-Weight Organic Acids

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The sorption and elution of glycolic, oxalic, pyruvic, gluconic, maleic, and *p*-toluenesulfonic acids using the strongly basic resin Dowex 2 (acetate form) and the weakly basic resin Dowex 3 (free-base form) have been studied. All acids except gluconic acid can be taken up quantitatively by the resins.

Glycolic, oxalic, gluconic, and maleic acids can be quantitatively displaced from both types of resins by means of sulfuric acid or ammonium carbonate. From the strongly basic resin, pyruvic acid can be displaced quantitatively only by means of acid elutriants. The elution of this acid from the weakly basic resin is in no case quantitative. Hydrochloric acid is the most effective elutriant for *p*-toluenesulfonic acid when the strongly basic resin is concerned, whereas the weakly basic resin is most easily regenerated with sodium hydroxide.

Several authors have used anion exchange resins for the isolation of organic acids. The results are summarized in Samuelson's monograph [1]. This topic has also been studied in some more recent papers [2-6]. The uptake of the acids does not seem to have caused any troubles whereas in some cases it has been reported that the recovery in the elution step is incomplete. Most authors have been interested in the chromatographic separation of different acids from each other. Very little attention has been given to the question of whether the recovery in the regeneration step can, from an analytical point of view, be considered as quantitative. For this reason it seemed to be appropriate to study in greater detail the uptake and especially the elution of different types of organic acids.

EXPERIMENTAL TECHNIQUE

Ion Exchange Column. The ion exchange column was of common construction (Ref.[1], Fig. 23). The diameter and length of the resin bed were 9 mm and 170 mm. The resin had the particle size 0.20—0.30 mm in an air-dry condition. The acetate form of the resin was prepared by passing 400 ml *M* sodium acetate solution through the column and sub-

sequent washing with water. In order to transform the resin into the free-base form it was instead treated with the same amount of *M* sodium hydroxide solution, precautions being taken to exclude carbon dioxide. Dowex 2 was regenerated according to the ordinary column technique whereas the regeneration of Dowex 3 was achieved by a combined batch and column operation to avoid channeling due to the swelling of the resin. Fifty ml of the elutriant was placed in the funnel attached to the column. After placing a stopper in the upper end of the funnel the column was shaken in order to transfer the resin into the funnel. The resin was then allowed to settle in the column and the elutriant passed through.

The resins are not quite stable in water and interference occurred because of dissolution of resin material. In order to eliminate as far as possible this source of error the resin was conditioned with 500 ml *M* sodium hydroxide solution and afterwards with 500 ml of the elutriant. The conditioning was repeated several times.

In all experiments the added amount of acid was about 2 meq and the flow rate about 2 ml/min. All figures given in the tables are expressed in per cent of the added amount.

Determination of the Acids. Glycolic, gluconic, and maleic acids were determined by oxidation with dichromate in sulfuric acid solution according to the following scheme.

$$\begin{aligned} C_2H_4O_3 + 3\ O &\longrightarrow 2\ CO_2 + 2\ H_2O \\ C_6H_{12}O_7 + 11\ O &\longrightarrow 6\ CO_2 + 6\ H_2O \\ C_4H_4O_4 + 6\ O &\longrightarrow 4\ CO_2 + 2\ H_2O \end{aligned}$$

The equivalent weights of glycolic, gluconic, and maleic acids are 1/6, 1/22 and 1/12 of the molecular weights, respectively.

The acid was introduced into a 1-liter flask and 0.5 *N* $K_2Cr_2O_7$-solution, to an excess of at least 1 meq, was added from a micro-buret. After addition of water to 60 ml volume, 100 ml conc. sulfuric acid was added under shaking. The flask was allowed to stand for 30 min and then cooled. After adding 500 ml distilled water, the flask was again cooled to room temperature. The excess potassium dichromate was determined in the usual manner by adding potassium iodide and titrating with sodium thiosulfate. In control experiments with the pure acids, the error was in all cases less than 0.5 %.

Oxalic acid was determined by oxidation with permanganate. Pyruvic acid was analyzed according to a colorimetric method [7]. *p*-Toluenesulfonic acid was determined spectrophotometrically in ultraviolet at 260 mμ. The spectrophotometric readings were made with a Beckman spectrophotometer model DU.

Blank experiments were run in order to obtain a correction for the dissolution of organic matters from the resin. The correction was in no case greater than 2 %.

RESULTS

The experiments with glycolic, oxalic, pyruvic, maleic, and *p*-toluenesulfonic acids show that all these acids can be taken up quantitatively by the acetate form of a strongly basic resin and by the free-base form of a weakly basic resin. The uptake of gluconic acid is incomplete with both types of resin. The amount of acid which passes through the column is indicated under the Tables 1 and 2. The fact that gluconic acid is not retained quantitatively is explained by the presence of lactones.

The results presented in Tables 1 and 2 show that glycolic, oxalic, gluconic, and maleic acids can be easily displaced from both types of resins by means of 0.5 *M* sulfuric acid or 0.5 *M* ammonium carbonate solutions. As can be seen from Table 3, oxalic acid is eluted with 150 ml of *M* HCl, 0.5 *M* H_2SO_4, 0.5 *M* $(NH_4)_2SO_4$, 0.5 *M* $(NH_4)_2CO_3$, or *M* NaOH.

The behavior of pyruvic and *p*-toluenesulfonic acids is much more complicated. Table 4 shows that pyruvic acid is recovered quantitatively from the strongly basic resin with hydrochloric acid or sulfuric acid, whereas unsatis-

Table 1. Elution of acids from the strongly basic resin Dowex 2 (acetate form).

Acid	Elution with 150 ml 0.5 *M* H_2SO_4 %		Elution with 150 ml 0.5 *M* $(NH_4)_2CO_3$ %	
Glycolic acid	99.0;	99.8	99.1;	99.5
Oxalic acid	99.2;	99.3	99.8;	100.2
Pyruvic acid	99.3;	99.9	55.9;	58.9
Gluconic acid *	76.8;	77.0	78.8;	76.6
Maleic acid	99.5;	100.1	99.7;	99.9
p-Toluenesulfonic acid	1.2;	2.0	0.0;	0.0

* The amount of gluconic acid in the effluent from the sorption step was 22.8, 23.0, 23.1, and 23.1 % respectively.

Table 2. Elution of acids from the weakly basic resin Dowex 3 (free-base form).

Acid	Elution with 150 ml 0.5 *M* H_2SO_4 %		Elution with 150 ml 0.5 *M* $(NH_4)_2CO_3$ %	
Glycolic acid	99.0;	99.4	99.1;	99.7
Oxalic acid	99.4;	100.2	99.3;	99.7
Pyruvic acid	24.2;	30.9	27.6;	28.9
Gluconic acid *	79.4;	78.6	79.6;	78.5
Maleic acid	100.0;	100.1	100.1;	100.1
p-Toluenesulfonic acid	25.6;	27.8	65.3;	68.9

* The amount of gluconic acid in the effluent from the sorption step was 20.1, 20.7, 20.1, and 20.6 % respectively.

Table 3. Elution of oxalic acid from the strongly basic resin Dowex 2 (acetate form) and the weakly basic resin Dowex 3 (free-base form) with 150 ml of different elutriants.

Elutriant	Elution from Dowex 2 %		Elution from Dowex 3 %	
M HCl	99.4;	100.0	99.7;	100.3
0.5 *M* H_2SO_4	99.4;	99.3	99.4;	100.2
0.5 *M* $(NH_4)_2SO_4$	99.7;	99.9	100.0;	100.1
0.5 *M* $(NH_4)_2CO_3$	99.8;	100.2	99.6;	99.7
M NaOH	100.1;	100.1	99.8;	100.1

factory results are obtained with the other elutriants. From the free-base form of the weakly basic resin, pyruvic acid can be removed only to a limited extent even when large quantities and high concentrations of the elutriants are used.

Table 4. Elution of pyruvic acid from the strongly basic resin Dowex 2 (acetate form) and the weakly basic resin Dowex 3 (free-base form).

Elutriant	Elution from Dowex 2 %		Elution from Dowex 3 %	
150 ml *M* HCl	99.6;	99.8	29.1;	34.3
300 ml *M* HCl	—		20.7;	29.5
500 ml *M* HCl	—		35.0;	36.8
150 ml 3 *M* HCl	—		24.6;	26.4
150 ml 0.5 *M* H_2SO_4	99.3;	99.9	24.4;	30.9
300 ml 0.5 *M* H_2SO_4	—		21.7;	24.6
500 ml 0.5 *M* H_2SO_4	—		32.9;	35.0
150 ml 1.5 *M* H_2SO_4	—		26.4;	26.4
150 ml 0.5 *M* $(NH_4)_2SO_4$	91.0;	94.8	23.6;	26.0
300 ml 0.5 *M* $(NH_4)_2SO_4$	88.9;	89.7	23.8;	24.6
500 ml 0.5 *M* $(NH_4)_2SO_4$	89.0;	89.9	30.5;	32.3
150 ml 1.5 *M* $(NH_4)_2SO_4$	86.4;	90.6	9.4;	12.4
150 ml 0.5 *M* $(NH_4)_2CO_3$	55.9;	58.9	27.6;	28.9
300 ml 0.5 *M* $(NH_4)_2CO_3$	63.0;	64.6	17.7;	24.6
500 ml 0.5 *M* $(NH_4)_2CO_3$	68.3;	70.9	33.7;	36.4
150 ml 1.5 *M* $(NH_4)_2CO_3$	32.7;	35.4	16.5;	19.7
150 ml *M* NaOH	82.7;	84.6	21.1;	30.9
300 ml *M* NaOH	84.6;	86.6	27.0;	27.0
500 ml *M* NaOH	85.0;	86.4	39.4;	40.6
150 ml 3 *M* NaOH	63.0;	66.9	14.8;	20.7

In separate experiments it was observed that the strongly basic resin, used for the uptake of pyruvic acid and subsequently treated with ammonium sulfate, could not be regenerated with hydrochloric acid. No traces of pyruvic

Table 5. Elution of p-toluenesulfonic acid from the strongly basic resin Dowex 2 (acetate form) and the weakly basic resin Dowex 3 (free-base form).

Elutriant	Elution from Dowex 2 %		Elution from Dowex 3 %	
150 ml *M* HCl	0.8;	2.2	21.9;	24.1
300 ml *M* HCl	32.4;	33.8	44.4;	46.8
500 ml *M* HCl	70.1;	72.8	71.3;	72.9
150 ml 0.5 *M* H_2SO_4	1.2;	2.0	25.6;	27.8
300 ml 0.5 *M* H_2SO_4	10.1;	11.4	44.0;	45.0
500 ml 0.5 *M* H_2SO_4	40.5;	41.3	62.0;	63.6
150 ml 0.5 *M* $(NH_4)_2SO_4$	0.0;	0.0	25.7;	29.1
300 ml 0.5 *M* $(NH_4)_2SO_4$	0.0;	0.0	35.8;	36.6
500 ml 0.5 *M* $(NH_4)_2SO_4$	0.0;	0.0	44.6;	45.5
150 ml 0.5 *M* $(NH_4)_2CO_3$	0.0;	0.0	65.3;	68.9
300 ml 0.5 *M* $(NH_4)_2CO_3$	0.0;	0.0	76.3;	79.4
500 ml 0.5 *M* $(NH_4)_2CO_3$	0.0;	0.0	82.9;	86.8
150 ml *M* NaOH	0.6;	1.4	94.9;	98.6
300 ml *M* NaOH	7.3;	9.2	99.9;	100.0
500 ml *M* NaOH	29.3;	30.6	—	

Table 6. Elution of p-*toluenesulfonic acid from the strongly basic resin Dowex 2 (acetate form) with 250 ml hydrochloric acid of different concentration.*

Elutriant concentration	Elution %
1 *M*	39.3
2 *M*	61.6
3 *M*	81.5
4 *M*	83.4
6 *M*	81.3

acid could be detected in the effluent from the regeneration step when the solution was investigated by the colorimetric method. Similar experiments in which the resin was treated with ammonium carbonate or sodium hydroxide instead of ammonium sulfate gave the same results. In some experiments with pyruvic acid, the determinations were made by an alkalimetric titration. The results indicate that the incomplete recovery of pyruvic acid is due to a decomposition of the acid during the ion exchange cycle.

The data given in Table 5 show that, for the elution of *p*-toluenesulfonic acid from the strongly basic resin, hydrochloric acid is more effective than sulfuric acid and sodium hydroxide. It is interesting to note that no traces of the acid have been detected in the effluent after treatment with large amounts of ammonium sulfate and ammonium carbonate solutions in the elution step. The influence of the strength of hydrochloric acid used as elutriant for *p*-toluenesulfonic acid has been studied. The results are given in Table 6. As can be seen from the table, 3 *M* hydrochloric acid is an effective elutriant. As a complement to the data presented in the tables two elutions with 500 ml 3 *M* hydrochloric acid were performed. The recovery was 100.0 % in both experiments.

From the weakly basic resin, *p*-toluenesulfonic acid is eluted quantitatively by means of sodium hydroxide solution (Table 5). The other elutriants investigated are less effective and are unsatisfactory for practical use.

Separate experiments show that *p*-toluenesulfonic acid is not fixed in an irreversible manner by any of the elutriants used in the present investigation. After the passage of 150 ml of the elutriants giving an incomplete elution from the strongly basic resin, a quantitative displacement is achieved on subsequent treatment with 500 ml 3 *M* hydrochloric acid. In similar experiments with the weakly basic resin a complete recovery is obtained by final displacement with 300 ml *M* sodium hydroxide.

As already mentioned gluconic acid is not taken up quantitatively under the conditions used in the experiments described above. A quantitative uptake of gluconic acid can be achieved by passing a solution of the sodium salt through a column filled with a strongly basic resin in the acetate form. From an acid solution, gluconic acid can be conveniently taken up by shaking the solution with an excess of the free-base form of the weakly basic resin for 24 h. The acid can then be eluted completely with 150 ml 0.5 *M* H_2SO_4 from both the strongly basic and the weakly basic resin.

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Alternative Mechanisms of the Fe- and the Cu-Fe-catalyzed Decomposition of Hydrogen Peroxide

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To describe the kinetic results of Sten Andersen [1,2] new schemes of reactions are proposed which agree better than his own schemes with the ideas of other authors regarding feasibility of the assumed intermediates. The schemes include a ferro-ferric or a cupro-cupric cycle, and the correct functional form of the kinetic equations is obtained through moderation of the cyclic reaction not as usual through an equilibrium, but through another irreversible cyclic process, which is considered slow in comparison with the total rate. Free radicals seem to appear in the cycles only bound to the metal ions.

The recent work of Andersen [1,2] has greatly increased our knowledge of the rates of decomposition of hydrogen peroxide at varying concentrations of ferric, cupric and hydrogen ions. Arguing from his results Andersen and Christiansen [3] have maintained the impossibility of a cyclic mechanism involving the shift of ferric to ferrous ions and hydroxyl radicals as has been first proposed by Haber and Weiss. The object of the present paper is to show, that although this closed sequence as well as other previous ones are in disagreement with experimental results, it is nevertheless possible to construct one which fits just as well as the open sequence of Andersen [1]. Several features in the general nature of the reaction indicate a closed sequence, a chain reaction. The inhibitor action of several organic compounds is the most obvious.

The scheme to be proposed below shares with schemes by Weiss, by Barb, Baxendale, George and Hargrave and by Uri the desirable feature that shifts in electron spin pairing occur only when mediated by iron or copper. The disagreement between these previous schemes and Andersen's experiments is overcome mainly through that further assumption that free radicals in the presence of cupric and ferric ions will be so strongly bound to these that they will appear as $FeOH^{+++}$, FeO^{++}, $OFeOH^{+}$, $CuOH^{++}$, CuO^{+} and similar ions. This feature had to be introduced in order to obtain the right dependence of rate constants on ferric ion concentration. It seems not unlikely that such complexes might be relatively stable due to resonance between structures with tetravalent and trivalent iron.

The fact that reaction of hydrogen peroxide with ceric ions leads only to reduction of ceric ions and not to catalytic decomposition is in accordance with the assumption of ferric-complexes as intermediates in preference to free hydroxyl radicals, as the latter might just as well be formed through reaction with ceric as with ferric ions.

The results reported from experiments with ^{18}O labelled hydrogen peroxide [4] require that the final evolution of oxygen (reaction 31 below) takes place as a dehydrogenation of H_2O_2, *e.g.* through a hydride ion (H^-) transfer plus dissociation of a proton (*cf.* the enzymatic dehydrogenation according to Westheimer and Vennesland [5]).

THE FERRO-FERRIC CYCLE

The cycle to be proposed is:

$$Fe^{++} + \dot{F}eO_2H^{++} \rightleftharpoons Fe\dot{F}eO_2H^{+4} \qquad 12$$

$$Fe\dot{F}eO_2H^{+4} \rightleftharpoons \dot{O}\dot{F}eOH^{+} + \dot{F}e^{+++} \qquad 23$$

$$\dot{O}\dot{F}eOH^{+} + H_2O_2 \rightarrow Fe^{++} + \dot{O}_2 + OH^- + H_2O \qquad 31$$

The level at which this cycle operates is determined by a "moderator cycle", consisting of two irreversible reactions 0.33′ and 12.0 together with 31 and the protolysis of the ferric hexaquo ion:

$$\dot{F}eOH^{++} + H_2O_2 \rightleftharpoons \dot{F}eO_2Haq^{++} \rightarrow \dot{O}\dot{F}eOH^{+} + \dot{O}H + H^+ \qquad 0(0')33'$$

$$\dot{F}eaq^{+++} + \dot{O}H \rightleftharpoons \dot{O}\dot{F}eOH^{+} + 2H^+ \qquad 3'3$$

$$Fe^{++} + Fe\dot{F}eO_2H^{+4} + H_2O \rightarrow 3\ \dot{F}eOH^{++} \qquad 12.0$$

$$\dot{F}eaq^{+++} \rightleftharpoons \dot{F}eOH^{++} + H^+ \qquad ie$$

Reaction 0.33′ starts with the formation of the wellestablished brown ferric perhydroxo complex in equilibrium with ferric ions, hydrogen ions and hydrogen peroxide. The rate determining step is an excitation of this complex ion and we picture it here as a splitting off of a hydroxyl radical which immediately afterwards attaches itself to a ferric ion. Bimolecular reaction between the complex ion and ferric ion to form two active particles would give a different kinetic expression. The ion $FeFeO_2H^{+4}$ is a perhydroxo dimeric analogue of the well-known polynuclear ions which in alkaline solution grow to colloid or macroscopic size. It is presumably stabilized by valence shift between the iron atoms.

The ion written as $OFeOH^+$ might for its composition be a ferro perhydroxo complex, and kinetic data obviously cannot exclude that. That would be rather unlikely, however, in consideration of its role in the scheme.

Reaction 12 and 23 can be interpreted so, that $OFeOH^+$ is formed from Fe^{++} and H_2O_2 catalyzed by $FeOH^{++}$, and it seems not very likely that the interchange of a coordinated water molecule with hydrogen peroxide should be much promoted by this catalyst. Furthermore, the only significant way of deactivation is 12.0 and the only feasible reason why a reaction 13.0 between Fe^{++} and $OFeOH^+$ does not contribute to the deactivation is that $OFeOH^+$ is the most energy-rich of the three cyclic reactants and therefore the most rare. This is in accordance with our assumption below that 12 is virtually in equilibrium.

The basic physical event which has to occur during the cycle is the splitting up of one pair of electrons with mutually compensating spin into two single electrons with parallel spin corresponding to the change from diamagnetic hydrogen peroxide to paramagnetic oxygen. Is this splitting up most likely to occur in 12, in 23 or in 31? To help the general views, dots have been placed to indicate unpaired electrons, the first four on iron being

omitted. In 23 a ferric ion leaves the binuclear complex with one electron less than the ferro ion brought into it in 12, and yet with one more unpaired. Hence 23 is the obvious place for the actual splitting and $OFeOH^+$ is most likely to have six unpaired electrons and no O—O bond.

On the other hand, the total back reaction 32, 21, 0'0 leads to some exchange of oxygen between water and peroxide, and the results of Cahill and Taube [4] may force us to assume that the O—O bond is first broken in the irreversible 31 reaction.

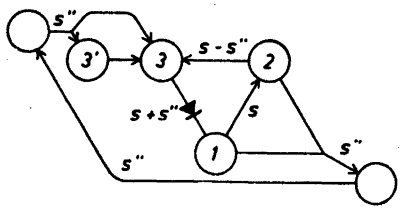

Fig. 1.

The diagrammatic representation of the reaction cycle with the active intermediates X_1, X_2, X_3 and X_3, representing Fe^{++}, $FeFeO_2H^{+4}$, $OFeOH^+$, and OH is given in Fig. 1.

We have supplemented Christiansens way of drawing such flow diagrams by the introduction of the symbol of a rectifier corresponding to the irreversible step. It is easily seen that the kinetic equations depend on where this step is placed in the cycle. In the work with a problem like the present one it is very convenient if the diagram unambiguously characterizes a scheme of reactions to which a certain set of kinetic expressions can be attached.

To comply with stationarity, the flow in the reactions 0.33' and 12.0 where two active particles appear or disappear, has to be the same: $s_{0.33'} = s_{12.0} = s_i''$, and this flow is considered negligible compared to the flow, s_i, in the oxygen producing cycle:

$$s_{31} = s_i + s_i'' \simeq s_{12} = s_i \simeq s_{23} = s_i - s_i'' \qquad (1)$$

Proceeding as Christiansen we obtain the following equations between the concentrations x_1, x_2, x_3 of the intermediates, X_1, X_2, X_3, the concentration of ferric ion, c_i, and of $FeOH^{++}$, c_e; the probabilities w_{ij} of the intermediate X_i to react under formation of X_j, the corresponding rate constants, k_{ij}, and the H_2O_2 concentration, x. K_i is the dissociation constant of $Feaq^{+++}$ (reaction ie), K_0 the equilibrium constant of the hydrolyzed ferric perhydroxo complex (reaction 00').

$$\begin{aligned} s_i &= x_1w_{12} - x_2w_{21} \\ &= x_2w_{23} - x_3w_{32} \\ &= x_3w_{31} \end{aligned} \qquad (2) \qquad \begin{aligned} s_i'' &= w_{0.33'} = k_{0.33'}\, c_e x \\ &= w_{12.0} = k_{12.0}x_1x_2 \end{aligned} \qquad (3)$$

(2) and (3) express that there is stationarity: the same net flow all through in each of our cycles. The equations (2) are solved with respect to x_1, x_2 and x_3:

$$x_1/s_i = (1 + w_{21}/w_{23}(1 + w_{32}/w_{31}))/w_{12} \qquad (4)$$

$$x_2/s_i = (1 + w_{32}/w_{31})/w_{23} \qquad (5)$$

$$x_3/s_i = 1/w_{31} \qquad (6)$$

The unknown concentrations of the intermediates, x_1, x_2 and x_3 are eliminated between (3), (4), (5) and (6), giving:

$$\begin{aligned}\frac{1}{s_i^2} &= \frac{k_{12.0}}{w_{0.33'}} \cdot \frac{x_1}{s_i} \cdot \frac{x_2}{s_i} \\ &= \frac{k_{12.0}}{w_{0.33'} w_{12} w_{23}} \left(1 + \frac{w_{21}}{w_{23}} \left(1 + \frac{w_{32}}{w_{31}}\right)\right) \left(1 + \frac{w_{32}}{w_{31}}\right) \\ &= \frac{k_{12.0}}{k_{0.33'} c_e x k_{12} K_0 c_e x k_{23}} \left(1 + \frac{k_{21}}{k_{23}} \left(1 + \frac{k_{32} c_i}{k_{31} x}\right)\right) \left(1 + \frac{k_{32} c_i}{k_{31} x}\right).\end{aligned} \tag{7}$$

If $k_{21} \gg k_{23}$ we get the same result as we would have obtained by assuming equilibrium in reaction 12 ($k_{12}/k_{21} = K_{12}$):

$$-\frac{dt}{d\ln x} = \frac{x}{2s_i} = \frac{A_i}{c_i} \left(1 + \frac{k_{32} c_i}{k_{31} x}\right) \tag{8}$$

$$A_i = \frac{c_{H^+}}{2k_{23} K_i} \sqrt{\frac{k_{12.0} k_{21}}{k_{0.33'} k_{12} K_0}}$$

with the required functional dependence on c_{H^+}, c_i and x. The formulae of the various ions appearing in the cycle are to some extent arbitrary as any of the steps may cover details which do not influence the kinetic equations, *e.g.* changes in the protolytic state or in aquotisation. By no means any change in this respect is permissible, however, but any scheme will cover an appreciable number of varieties.

To compare the scheme presented here with previous ones, we may consider Christiansen's calculations on a free radical-ferro-ferric cycle (Ref. 3, p. 1060). In that case the level at which the cycle works is moderated through an equilibrium of two cyclic reactants with energy-rich peroxide:

$$\dot{F}eOH^{++} + H_2O_2 \rightleftharpoons Fe^{++} + H\dot{O}_2 + H_2O$$

and it is proved that this leads to an equation of the form

$$-x \frac{dt}{dx} = -\frac{dt}{d\ln x} = A_1 \sqrt{1 + \beta/x} \tag{9}$$

while the experiments require:

$$-\frac{dt}{d\ln x} = A_1 \left(1 + \frac{\alpha}{x}\right). \tag{10}$$

In a similar way the present cycle leads to a mono-molecular proces ($\alpha = 0$) if it is moderated by reaction 0.33′ in equilibrium, $w_{12.0}$ being zero. In both cases it is seen that $-dt/d\ln x$ increases too slowly or not at all as the reaction proceeds (falling values of x). In the derivations above this is corrected through the combination of the irreversible 0.33′ production of active intermediates with the irreversible 12.0 destruction of activity. This makes the level sink more rapidly with falling H_2O_2 concentration. In consideration of the energy levels through which the "moderator cycle" 0.33′, 3′3, (31 + 32), 12.0 will be running, it seems reasonable that the reverse reactions 33′.0 and 0.12 can safely be neglected ($w_{0.12} \ll w_{0.33'}$; $w_{33'.0} \ll w_{12.0}$).

THE PROMOTING ACTION OF COPPER

Adopting the scheme above for the ferric-ion catalysis we need not restrict ourselves to the very limited class of reactions considered by Andersen for the description of the copper promotion, and possibilities are opened to get through without introduction of entropy producing *perpetuum mobile* reactions as Andersen's cycle: 12—23—33′—3′1 in his final scheme (his symbols).

We will not attempt to reproduce Andersen's results in the same mathematical form, which is adapted to his special class of reactions. We fix our attention to the fact which appears directly from the data given in Table 2, Ref. 2, that at copper ion concentrations greater than 0.0004 his A_1 values become practically independent of copper concentration, while at a given copper concentration, A_1 tends to become proportional to the square root of iron concentration. At the same time A_2/A_1, corresponding to α in equation (10), grows independent of iron concentration. In copper-free solutions A_2/A_1 was proportional to iron concentration such as to make A_2 itself independent of iron.

To obtain a pattern of reactions which corresponds to the behavior at *high Cu-concentrations*, we will assume the initiating reaction 0.33′ to be unchanged by the presence of copper. Once formed the radical activity is supposed to be taken over by copper intermediates through the equilibrium 36 in which the ions on the right hand side are supposed to predominate:

$$\dot{O}\dot{F}eOH^{+} + \dot{C}u^{++} \rightleftharpoons \dot{F}eOH^{++} + \dot{C}u\dot{O}^{+} \qquad 36$$

CuO^+ is called X_6 and is part in a copper cycle 4564 in which X_4 is Cu^+ and X_5 is $CuO_2H_2^+$:

$$Cu^{+} + H_2O_2 \rightleftharpoons CuO_2H_2^{+} \qquad 45$$

$$CuO_2H_2^{+} \rightleftharpoons \dot{C}u\dot{O}^{+} + H_2O \qquad 56$$

$$\dot{C}u\dot{O}^{+} + H_2O_2 \rightarrow Cu^{+} + \dot{O}_2^{\cdot} + H_2O \qquad 64$$

and deactivation occurs through:

$$Cu^{+} + CuO_2H_2^{+} \rightarrow 2\ \dot{C}u^{++} + 2OH^{-} \qquad 45.0$$

Neglecting the iron cycle 1231 we obtain equations identical with the previous ones when 1, 2 and 3 are substituted by 4, 5 and 6 except in $w_{0.33'}$, which is kept unchanged:

$$x_4/s_u = (1 + w_{54}/w_{56}(1 + w_{65}/w_{64}))/w_{45} \qquad (11)$$

$$x_5/s_u = (1 + w_{65}/w_{64})/w_{56} \qquad (12)$$

$$x_6/s_u = 1/w_{64} \qquad (13)$$

$$\frac{1}{s_u^2} = \frac{k_{45.0}}{w_{0.33'}w_{45}w_{56}}\left(1 + \frac{w_{54}}{w_{56}}\left(1 + \frac{w_{65}}{w_{64}}\right)\right)\left(1 + \frac{w_{65}}{w_{64}}\right) = \frac{k_{45.0}}{k_{0.33'}c_e x k_{45} x k_{56}}\left(1 + \frac{k_{54}}{k_{56}}\left(1 + \frac{k_{65}}{k_{64}x}\right)\right)\left(1 + \frac{k_{65}}{k_{64}x}\right) \qquad (14)$$

and if $k_{54} \gg k_{56}$:

$$-\frac{dt}{d\ln x} = \frac{x}{2s_u} = \frac{A_u}{\sqrt{c_i}}\left(1 + \frac{k_{65}}{k_{64}x}\right) \tag{15}$$

$$A_u = \frac{1}{2k_{56}}\sqrt{\frac{k_{45.0}k_{54}}{k_{0.33'}k_{45}K_i}} \cdot \sqrt{c_{H^+}}$$

We have no experimental evidence to show whether the dependence on c_{H^+} is right or wrong, and it is easily changed by changing the protolytic state of the reactants.

The dependence on the square root of ferric ion concentration and the independence of copper concentration are as required, but whereas the square root dependence may safely be taken as evidence for the reality of a closed-sequence iron cycle, too much weight should certainly not be laid upon the way in which copper ions enter the present scheme.

It makes no difference in the final expression if 45 is catalyzed by ferric ion, as we have actually assumed equilibrium in this step. 45 may therefore be substituted by for instance:

$$Cu^+ + FeO_2H^{++} \rightleftharpoons CuO_2H_2^+ + FeOH^{++} \qquad 45\ (a)$$

In the region of *low Cu-concentrations* we must take both cycles into consideration. This can be done in several ways, but in order not to make things too complicated we will stick to the class of reactions already laid out and add only two reactions:

$$Fe^{++} + CuO_2H_2^+ \rightarrow \dot{F}eOH^{++} + \dot{C}u^{++} + OH^- \qquad 15.0$$

$$Cu^+ + Fe\dot{F}eO_2H^{+4} + H_2O \rightarrow \dot{C}u^{++} + 2\dot{F}eOH^{++} + OH^- \qquad 42.0$$

They are deactivation reactions and their velocities are s_{iu}'' and s_{ui}''.

With reaction 36 in equilibrium we have: $x_6c_e = K_{36}x_3c_u$ and when s_i and s_u are the rates of the two generating cycles (*cf.* eq. 2):

$$s_i = x_3w_{31} \qquad s_u = x_6w_{64} \qquad s = s_u + s_i \tag{16}$$

$$\frac{s_u}{s_i} = \frac{k_{64}K_{36}c_u}{k_{31}c_e} = \frac{\gamma c_u}{c_i} \qquad (\gamma = \gamma_0 c_{H^+}) \tag{17}$$

$$\frac{1}{s} = \frac{1}{s_i}\,\frac{1}{1 + \gamma c_u/c_i} \tag{18}$$

For the moderator cycle we now get (*cf.* eq. 3):

$$\begin{aligned} s'' &= w_{0.33'} = k_{0.33'}\, c_e x \\ &= s_i'' + s_{ui}'' + s_{iu}'' + s_u'' \\ &= k_{12.0}x_1x_2 + k_{15.0}x_1x_5 + k_{42.0}x_4x_2 + k_{45.0}x_4x_5 \end{aligned} \tag{19}$$

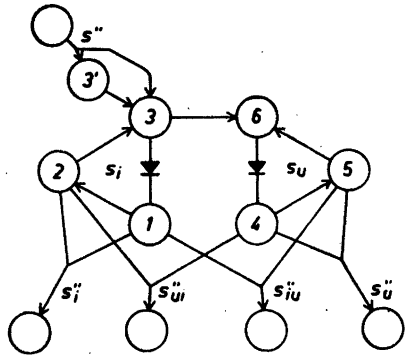

Fig. 2.

We still neglect s'' in comparison with s_i and s_u. Equations 4, 5, 6, 11, 12 and 13 then remain valid, and we can derive the following equations in a similar way and under similar conditions as before ($k_{21} \gg k_{23}$ and $k_{54} \gg k_{56}$):

$$\frac{1}{s^2} = \frac{1}{w_{0.33'}\,(1+\gamma c_u/c_i)^2}\left\{k_{12.0}\frac{x_1}{s_i}\frac{x_2}{s_i} + \frac{\gamma c_u}{c_i}\left(k_{15.0}\frac{x_1}{s_i}\frac{x_5}{s_u} + k_{42.0}\frac{x_4}{s_u}\frac{x_2}{s_i}\right) + \left(\frac{\gamma c_u}{c_i}\right)^2 k_{45.0}\frac{x_4}{s_u}\frac{x_5}{s_i}\right\}$$

$$= \frac{4}{(1+\gamma c_u/c_i)^2}\frac{1}{x^2}\left\{\frac{A_i^2}{c_i^2}\left(1+\frac{k_{32}c_i}{k_{31}x}\right)^2 + \frac{\gamma c_u}{c_i}\left(\frac{A_{iu}^2}{c_i^2}+\frac{A_{ui}^2}{c_i}\right)\left(1+\frac{k_{32}c_i}{k_{31}x}\right)\left(1+\frac{k_{65}}{k_{64}x}\right) + \left(\frac{\gamma c_u}{c_i}\right)^2\frac{A_u^2}{c_i}\left(1+\frac{k_{65}}{k_{64}x}\right)^2\right\}$$

$$A_{iu}^2 = \frac{k_{15.0}k_{21}}{4\,k_{0.33'}K_0k_{12}k_{23}k_{56}K_i^2}\,c_H^2 + \qquad (20)$$

$$A_{ui}^2 = \frac{k_{42.0}k_{54}}{4\,k_{0.33'}K_ik_{45}k_{23}k_{56}}\,c_H +$$

At given concentrations of iron and copper this gives an expression of the form

$$-\frac{dt}{d\ln x} = A_1\sqrt{1+\beta/x+\alpha^2/x^2} \qquad (21)$$

Only when $\beta = 2\alpha$ will this reduce to the form of (10), but in quite wide intervals it will not be very far off. The exclusive ability of the integrated equation (10) to represent the experimental results appears to be somewhat overestimated by Andersen. In the published results of a typical experiment with both copper and ferric ions (Ref. 2, Table 1) the deviations between calculated and experimental values vary in a systematic way culminating at 71 min. with —0.84 min.

The possibility of choosing $\beta \neq 2a$ introduces one more parameter and must invariably make it possible to obtain a better agreement with a given experiment. To test whether the obtained values of the parameters are in agreement with our expression we would need the complete experimental material, but only one series of measurements is published in full.

It may be of some interest in judging the significance of an obtained agreement between observed and calculated values in kinetic experiments in general to print here a table of deviations from various integrals to equation (21) corresponding to different values of $\beta/a = n$:

$$t = y_n = \int_{x_0}^{x} A_1 \sqrt{1 + na/x + a^2/x^2}\ \mathrm{dlog}\ (a/x) \qquad (22)$$

The chosen values of the parameters are:

1. $n = 3$, $A_1 = 14.37$, $\log a/x_0 = \bar{1}.050$
2. $n = 3$, $A_1 = 14.59$, $\log a/x_0 = \bar{1}.035$
3. $n = 1$, $A_1 = 16.50$, $\log a/x_0 = \bar{1}.120$

and for comparison Andersen's parameter values, which correspond to:

4. $n = 2$, $A_1 = 15.29$, $\log a/x_0 = \bar{1}.056$

Table 1. $\triangle = t_{calc.} - t_{obs.}$

$t_{obs.}$ min.	1. (y_3)	2. (y_3)	3. (y_1)	4. (y_2, St. A.)
0	.00	.00	.00	.00
2.10	.00	+.03	+.12	+.03
5.00	−.12	−.08	+.06	−.02
10.00	−.20	−.10	+.15	−.03
15.00	−.08	+.03	+.32	+.10
20.00	−.05	+.05	+.30	+.12
25.00	−.01	+.15	+.24	+.11
30.00	+.25	+.46	+.35	+.26
35.00	+.28	+.38	+.16	+.21
43.00	+.32	+.51	−.16	+.10
50.00	+.18	+.56	−.36	+.05
60.00	+.29	+.58	−.58	−.14
71.00	−.28	−.27	−1.14	−.84
80.00	−.20	−.20	−.86	−.69
90.00	+.27	+.26	+.23	+.01
100.00	−.32	−.44	+.45	−.45
105.00	.00	−.25	+1.13	+.06
$\Sigma\triangle^2$	0.72	1.75	4.3	1.56 min^2

The integral (22) can be solved in terms of the ordinary functions and the choice between using the solutions or using a numerical integration becomes a matter of taste and of available calculating machines. The values of Table 1 were actually calculated by numerical methods in the following way [6]:

a. $f = \sqrt{1 + na/x + a^2/x^2}$ was tabulated at intervals of 0.1 in $\log a/x$ for $n = 1$ and $n = 3$ and controlled through the differences of first, second, and third order f^1, f^2, f^3.
b. From a plot of the experimental values of t *versus* $-\log x$ was found where $-dt/d\log x$ had increased to twice the value at $t = 0$.
c. In each of the tables of f is found where f doubles up within a similar interval in $\log a/x$.
d. Starting at the beginning of this interval the sums of first order ^1f were formed and corrected to form a table of the integral.
e. A_1 was chosen to fit the initial slope.
f. $\log a/x_0$ was chosen such as to make a fit at $t = 100$ min.

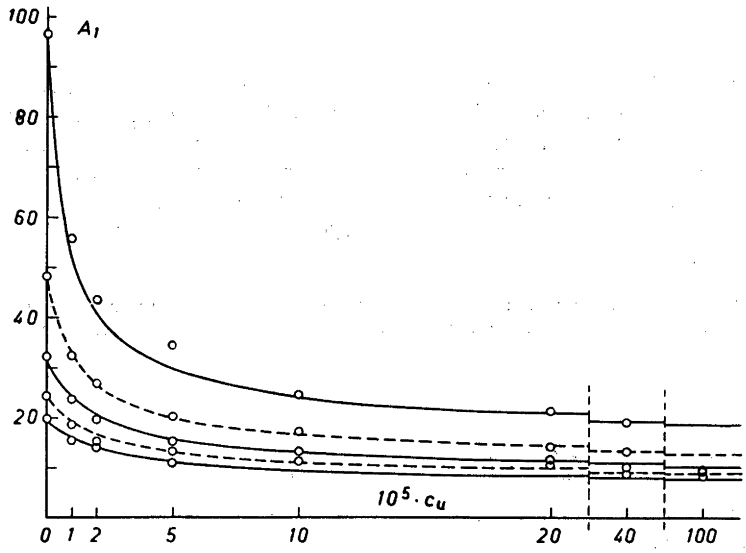

Fig. 3. Sten Andersen's experimental values of A_1 *(points) compared with curves calculated from eq. (23).*

g. The agreement was improved by trial and error with small changes in A_1 and log a/x_0.
h. For the final choice of parameters values were interpolated in the tabulated values of the integral using the f^1 and f^2 values as values of the differences of second and third order.

This procedure was adopted in order to obtain results which were directly comparable with Andersen's, although undue weights are actually placed on the initial measurements.

Equation (20) contains many adjustable parameters. Until experimental data from a wider range of iron concentrations and with variation of acidity appear, the only thing in the way of investigating quantitatively the applicability of (20) that can feasibly be done is to consider the constant A_1. If we suppose that it does not influence the value of A_1 very much whether it is chosen to fit an integral derived from (21) or from (10), we can take the experimental values from Table 2, Ref. 2, and try to fit them into the form required by (20) and (21):

$$A_1 = \frac{1}{1 + \gamma c_u/c_i} \frac{A_i}{c_i} \sqrt{1 + (\delta + \varepsilon c_i)\gamma c_u/c_i + \mu c_i(\gamma c_u/c_i)^2} \qquad (23)$$

In Fig. 3 the points represent the experimental values (Table 2, Ref. 2) and the lines equation (23) with $A_i = 0.0965$, $\gamma = 143$, $\delta = 0{,}50$, $\varepsilon = 0$, $\mu = 0{,}032$. A better fit could be obtained with negative values of ε.

Other equations to fit A_1, but to which no kinetic scheme has been found, are:

$$A_1 = \frac{0.555}{\sqrt{c_i}} \frac{4.9 + 10^5 c_u}{28\sqrt{c_i} + 10^5 c_u} \qquad (24)$$

$$A_1 = \frac{0.555}{\sqrt{c_i}} \frac{1.38 + 10^5 c_u + 4.13/10^3 c_i}{0.75 + 10^5 c_u + 250\ c_i} \qquad (25)$$

CONCLUSIONS

The reaction with pure iron seems accounted for by the present scheme in a very satisfactory way. For the combined action of copper and iron the scheme is far from final, though it is probably to be preferred to Andersen's original schemes as basis for further investigations. A more general conclusion concerns the danger of placing too much confidence in a scheme of reactions because it fits the kinetic data, even though a great number of others have been ruled out.

Except for the calculations in the last part, the content of this paper was presented in an *ex auditorio* opposition at the formal defense of the thesis of V. Sten Andersen on June the 15th, 1954.

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On the Use of a Thallic-thallous System as a pH Indicator

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In a former paper Rancke-Madsen, Skarbye-Nielsen and Østergaard [1] have shown that the ceric-cerous redox system on the addition of a suitable redox indicator can be used as pH indicator.

In the present paper it has been shown that an indicator system can be prepared in a similar way by the thallic-thallous redox system upon addition of potassium iodide and starch. The result is an indicator with two transition intervals in the pH scale, one of which is clear, reproducible and reversible. The transitions take place within a narrow pH range. Further it has been shown that this indicator system can be used as an indicator in the titration of carbonate ion as a monovalent base.

THE INDICATOR SYSTEM

The redox potential in a solution of a thallic + thallous salt is determined by:

$$E = E_o + \frac{0.06}{2} \cdot \log \frac{[Tl^{+++}]}{[Tl^{+}]}$$

E_o is reported [2] to be 1.280 volt. In the above expression $[Tl^+]$ is independent of pH, whereas $[Tl^{+++}]$, owing to the hydrolysis of the thallic ions and a possible precipitation of the sparingly soluble thallic oxide, is strongly dependent on pH (except at low pH values) [2]. It is stated [3] that thallic oxide is precipitated within a pH-range of *ca.* 1—2.5.

A suitable redox indicator must be added to the thallic-thallous system in order that the resulting system may be used as a pH indicator. Potassium iodide + starch proved to be applicable as a redox indicator. On addition of potassium iodide thallous iodide is precipitated, for which reason $[Tl^+]$ in the above expression is dependent on the amount of iodide added; but the resulting $[Tl^+]$ is still independent of pH. Iodide-starch changes from colourless to blue at $E = 0.60$—0.65 volt, and again from blue to colourless at $E = ca.\ 0.90$ volt (in both cases at pH = 7) [4].

In the indicator system mentioned here E increases with decreasing pH, and both the transitions mentioned have been observed.

EXPERIMENTAL

Solutions: Thallous nitrate: A 0.060 *M* $TlNO_3$ solution was prepared by dissolving the calculated weight of the salt in an appropriate amount of water.

Thallic nitrate: Thallous nitrate was oxidized with chlorine in a basic solution to yield a precipitate of thallic oxide. This substance was dissolved in concentrated nitric acid, and this solution was diluted such that the resulting solution of thallic nitrate was *ca.* 2 *M* with respect to nitric acid. It was necessary to acidify the solution rather strongly in order to prevent extensive hydrolysis of the thallic ions.

The content of thallic salt in this solution was determined by thiosulphatimetric titration: To 25 ml of the solution 2 g of potassium iodide were added, causing the formation of a dark-coloured precipitate ($\rightarrow TlI + I_3$). Then 2 ml of 1 % starch solution and 15 ml of ligroine were added, after which titration was performed with standard 0.1 *N* sodium thiosulphate until the iodine colour disappeared from the ligroine phase. The advantage of using ligroine (instead of a more dense liquid such as chloroform) is that ligroine is lighter than the water phase, and the precipitate therefore does not interfere with observations made on the top layer. The observation of the transition in this titration is not possible unless ligroine or a similar substance is added. The solution prepared in this way was found to be a 0.0642 *M* $Tl(NO_3)_3$.

Potassium iodide: The concentration of iodide in the *ca.* 0.1 *M* solution used was determined by argentometric titration, using dichlorofluorescein as adsorption indicator.

Apparatus: Values of pH and *E* were measured by means of a "pH meter 22" (Radiometer, Copenhagen). Reference electrode: Calomel electrode.

Experimental technique: 1) In a 100 ml beaker the following mixture was made: 25 ml water, 3 ml 1 % starch, *ca.* 5 ml 0.1 *M* sodium hydroxide.

2) A thallic nitrate solution was added (from a semi-micro burette), causing the solution to turn deeply yellow. The thallic hydroxide formed must be supposed to be in colloid solution. The pH in this solution was now *ca.* 11—12.

3) Thallous nitrate solution was added (from semi-micro burette).

4) Potassium iodide solution was added (from semi-micro burette), causing the formation of a yellow precipitate of thallous iodide. The solution was a deep yellow.

5) Nitric acid (0.1 *M*) was then added dropwise until the first colour transition (blue iodine-starch colour) was reached. The solution before this transition was yellow and the colour changed from yellow past green to blue. The deep green colour (situated between yellowish green and bluish green) has been regarded as a transition (first transition) in the experiments described in this paper.

6) pH and *E* were measured.

7) Nitric acid (0.1 *M*) was again added dropwise until the blue colour disappeared (second transition).

8) pH and *E* were measured.

RESULTS

Using the above method, a number of experiments were performed in which the added quantities of thallic and thallous nitrate were constant, but the quantity of potassium iodide was varied. Table 1 shows the mean values found in some series of experiments. On the addition of a very small quantity of iodide (series 1) no transition took place. The addition of a slightly larger quantity of iodide (series 2) produced a just observable — but indistinct — colour transition. It was not until a still larger quantity of iodide (series 3) was added that clear and distinct transitions were observed. The individual experiments in series 3 are recorded in Table 2. The figures in the last four columns refer to the reproducibility of the transitions: When the equivalence point has been determined for the first time, a little 0.1 *M* sodium hydroxide was added, causing the colour to change back. Then some more 0.1 *M* nitric acid was added, and when the transition occurred the pH and *E* values were again

Table 1. Mean values of pH and E in 1st and 2nd transitions of the indicator system thallic-thallous to which were added potassium iodide and starch.

The series of experiments were carried out at the temperature $t°$. In all experiments 0.20 ml of 0.0642 *M* $Tl(NO_3)_3$ and 0.20 ml of 0.060 *M* $TlNO_3$ were used. This means that the solution contains 0.02568 milliequivalents Tl^{+++} as oxidizing agent. A 0.01078 *M* KI was used. The *E*-values were corrected in order to have them stated in millivolt relative to the normal hydrogen electrode.

Series of Expts.	$t°$	ml KI	= milli-equivalents I^-	1st transition		2nd transition	
				pH	*E*	pH	*E*
1	22	0.20	0.00216		—	—	—
2	25	0.40	0.00432	7.15	757	3.92	1 040
3	24	0.50	0.00539	7.27	754	3.94	1 020
4	22	1.00	0.01078	7.85	707	3.61	1 036
5	22	1.50	0.01617	8.28	679	3.12	1 050
6	22	2.00	0.02156	8.45	667	2.08	1 058
7	25	2.25	0.02426	8.46	664	1.64	1 062
8	25	2.30	0.02479	8.47	662	—	—
9	25	2.50	0.02695	8.53	659	—	—

determined. The first transition proved to be easily reproducible at exactly the same values of pH and *E*; this was the case in all the other series of experiments, in which analogous tests were made. As regards the second transition, this can be satisfactorily reproduced, but it takes place at a higher pH and a lower *E* value. The other series gave the same results. These results may be due to the fact that some iodine is irreversibly oxidized to a higher oxidation number that that of free iodine.

*Table 2. Individual experiments in series No. 3 (*cf. *Table 1).*

The pH and *E*-values at the 1st and 2nd transitions of the indicator system thallic-thallous to which were added potassium iodide and starch are given in the four first columns. The pH and *E*-values in the four last columns refer to the reproducibility of the transitions (*cf.* the text). $t = 24°$. In all the experiments 0.20 ml of 0.0642 *M* $Tl(NO_3)_3$, 0.20 ml of 0.060 *M* $TlNO_3$ and 0.50 ml of 0.01078 *M* KI were used. The *E*-values were corrected in order to have them stated in millivolt relative to the normal hydrogen electrode.

m = mean value. μ = uncertainty on the single determination. μ_m = uncertainty on the mean value of the determinations.

Expt. No.	1st transition		2nd transition		1st transition repeated		2nd transition repeated	
	pH	*E*	pH	*E*	pH	*E*	pH	*E*
1	7.30	750	3.93	1 022	—	—	—	—
2	7.30	750	3.80	1 039	—	—	4.45	983
3	7.30	752	3.78	1 037	—	—	4.11	1 005
4	7.35	751	3.90	1 028	—	—	4.18	1 008
5	7.21	760	3.91	1 029	7.25	754	—	—
6	7.17	761	4.20	990	7.32	750	—	—
7	7.24	760	4.20	983	7.27	751	—	—
8	7.20	760	3.91	1 022	7.21	755	—	—
9	7.30	749	4.00	1 020	7.25	755	—	—
10	7.30	750	3.81	1 030	—	—	4.10	1 000
m	7.27	754	3.94	1 020	7.26	753	4.21	999
μ	0.06	5	0.15	19	0.04	2	0.16	11
μ_m	0.02	2	0.05	6	0.02	1	0.08	6

The results of the rest of the series are shown in Table 1. The uncertainty in these series was in no case more unfavourable for the first transition than that stated for series of experiments No. 3. The first transition proved to be distinct, reproducible and reversible all the way through. The second transition on the other hand, became less and less clear. Already in series No. 6 it was difficult to observe when the last blue colouring disappeared. It also became more and more difficult to reproduce the second transition. As the second transition implies that all iodide has been oxidized to free iodine, an excess of iodide ions in proportion to thallic ions will involve that the second transition cannot take place. Thus, the use of more than 2.38 ml of the potassium iodide solution represents an excess, but even the use of 2.30 ml (series No. 8) caused the nonappearance of the second transition.

As will be noticed, the E values at the transitions for all the experiments were higher that those stated in the literature [4] at the transitions for iodine-starch: 0.60—0.65 volt and *ca.* 0.90 volt, respectively. This may be due to the fact that owing to the yellow colour of the solution an excess of acid must be added in order to produce a distinct transition of colour. The excess of acid brings about a lower pH and consequently a higher E value.

In order to investigate the influence of the concentrations of thallic and thallous ions on the results, some series of experiments were performed, the results of which are shown in Table 3. It is seen that particularly the first transition is not greatly influenced by minor alterations of the concentrations mentioned.

Table 3. The mean values of pH and E *at the 1st and 2nd transitions of the indicator system thallic-thallous with potassium iodide and starch added.*

$t = 24°$. 0.0642 M $Tl(NO_3)_3$, 0.060 M $TlNO_3$ and 0.01078 M KI were used. The E-values have been corrected in order to have them stated in millivolt relative to the normal hydrogen electrode.

Series of Expts. No.	ml $Tl(NO_3)_3$	ml $TlNO_3$	ml KI	1st transition pH	1st transition E	2nd transition pH	2nd transition E
4	0.20	0.20	1.00	7.85	707	3.61	1 036
10	0.30	0.30	1.00	7.87	701	3.28	1 057
11	0.30	0.20	1.00	7.86	703	3.51	1 054
5	0.20	0.20	1.50	8.28	679	3.12	1 050
12	0.30	0.20	1.50	8.20	682	3.24	1 063

TITRATION OF CARBONATE

The indicator system mentioned above (first transition) was tested in the titration of carbonate ion as monovalent (monobasic, monoacid) base:

$$CO_3^{--} + H^+ \rightarrow HCO_3^-$$

The equivalence point is at $pH = \frac{1}{2}(pk_1 + pk_2) = ca.$ 8.3, depending to a certain degree on the ionic strength.

EXPERIMENTAL

Solutions: Sodium carbonate: Dried, anhydrous Na_2CO_3 *p.a.* was weighed, and a solution of known concentration was prepared. The concentration was also checked by titration with standard 0.1 *M* nitric acid after carbon dioxide had been boiled out. Result: The solution made was found to be a 0.2471 *M* Na_2CO_3.

Nitric acid: The standard solution was a 0.1055 *M* HNO_3.

Thallous nitrate, thallic nitrate and potassium iodide: The solutions described earlier in this paper were used.

Experimental technique: Since the thallic nitrate solution used for the production of the indicator system is strongly acid, it was necessary to adjust the indicator system at the pH value required (*ca.* 8.3) before the addition of the sample (*in casu* the solution of sodium carbonate).

Production of the indicator system: 25 ml water, 3 ml 1 % starch, *ca.* 5 ml 0.1 *M* sodium hydroxide, 0.20 ml thallic nitrate (*i.e.* 0.013 millimole), 0.20 ml thallous nitrate (*i.e.* 0.012 millimole), and 1.50 ml potassium iodide were, as in the earlier experiments, brought to the first transition by means of 0.1 *M* nitric acid. According to previous experiments pH is equal to 8.28.

10.201 ml of 0.2471 *M* Na_2CO_3 was added, after which titration was carried out with 0.1055 *M* HNO_3 to the same transition; the end volume was *ca.* 70 ml. In this solution the ionic strength was different from that in the indicator system before the addition of sodium carbonate. An experimental investigation shows that at the equivalence point the pH = 8.20 in the final volume. However, experiments also show that the quantity of acid which has to be used in order to change pH in the solution of the indicator system from 8.30 to 8.10—8.20 is very small and equal to *ca.* 0.02 ml. This means that the error which is made when fixing the solution of the indicator system at pH = 8.28 instead of pH = 8.20 can be considered negligible.

RESULTS

a) After various experiments the above indicator system was found to be the one that gave the most detectable change of colour at the equivalence point pH = 8.20.

The colour changes were found by fixing the indicator system at pH = 8.28. Then a little 0.1 *M* sodium hydroxide was added. The solution was diluted with water, and then 0.1 *M* nitric acid was added dropwise until the final volume was *ca.* 70 ml. The following colour changes were observed:

pH > 8.45—8.50	yellow
pH = 8.45—8.50	incipient dark-colouring
pH = 8.25—8.30	faint green
pH = 8.20	green (point of equivalence)
pH = 8.10	bluish green
pH = 8.00—8.05	blue
pH = 7.90—7.95	deep blue

Table 4. Titration of carbonate ion as monovalent base.

Indicator system: Thallic-thallous with potassium iodide and starch added. Titrand: 10.201 ml of 0.2471 *M* Na_2CO_3. Titrator: 0.1055 *M* HNO_3. As real value is considered: 23.89 ml HNO_3.

m = mean value. μ = uncertainty on single determination. μ_m = uncertainty on the mean value of the determinations.

Expt. No.	ml HNO_3	Deviation from real value ml	%
1	23.88	−0.01	0
2	23.80	−0.09	0.4
3	24.05	+0.16	0.7
4	23.90	+0.01	0
5	23.83	−0.06	0.3
6	23.94	+0.05	0.2
7	23.68	−0.21	0.9
8	24.08	+0.19	0.8
9	23.75	−0.14	0.6
10	24.14	+0.25	1.0
11	23.90	+0.01	0
12	23.78	−0.11	0.5
13	24.12	+0.23	1.0
14	23.87	−0.02	0.1
15	23.99	+0.10	0.4
16	23.72	−0.17	0.7
m	23.90		
μ	0.14		
μ_m	0.04		

b) The known solution of carbonate was titrated visually to the above-mentioned green colour. Sixteen experiments were carried out each with the same quantity of carbonate. The results are given in Table 4. Since the solutions were all of the same known concentration, calculations can be made to the effect that 23.89 ml 0.1055 *M* HNO_3 is equivalent to the quantity of carbonate used. It will be seen that the mean values of the results of the experiments (23.90 ml) is in good agreement with this, and that the individual results are in the interval 23.89 ml $\pm$ 1 %. Whenever titrations are carried out quickly, there is a tendency to use too much acid, possibly because this causes a local evolution of carbon dioxide from the solution.

The titrations were repeated with new solutions with results corresponding to those mentioned above.

Therefore, the results show that the indicator system in question can be used for the titration of carbonate ion to hydrogen carbonate ion. The method is fairly accurate, but cannot be characterized as a quick method.

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On the Method of Continuous Variations

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The implications and the validity of the assumptions made in the theory and in the application of the method of continuous variations as used in complex chemistry have been examined, and the results of such applications from numerous references (22 of which are given in the article) have been compiled and compared to results obtained by different methods where possible. On the basis of this it appears that the method can in general not be considered very reliable and that results obtained by the method must in general be considered as indications rather than solutions. In particular, most of the complexity constants so obtained must be considered erroneous in cases where the ligand number exceeds one. The method of continuous variations is compared to J. Bjerrum's method of corresponding solutions, the latter being superior in that it, for systems to which it is applicable, furnishes quantitative results.

Since the invention of the method of continuous variations (in the following referred to as c.v.) and especially since the work of Vosburgh and Cooper [1] this method has been extensively used in investigations of complex compound formation in solution. Furthermore c.v. has been adopted by standard works on physico-chemical methods for the investigation of reactions in solution [2,3]. It has therefore been of interest to compile some of the data obtained by means of c.v. for a critical review of the implications and the validity of the method.

The invention of c.v. is usually ascribed to P. Job [4,5]. It deserves mentioning that the principles of the method were already outlined by Denison [6] in 1912.

We shall have to consider two different applications of the method:

1) The application to cases in which the reactants, M and A, form only one complex, whereby it is possible not only to establish the stoichiometric composition of the complex but also to calculate its formation constant.

2) The application to cases in which M and A form several complexes, whereby only the composition of (some of) the complexes can be determined.

In the following discussion C denotes total concentrations, [A] the actual concentration of species A and x the "mole fraction" $\frac{C_A}{C_M + C_A}$.

FORMATION OF ONE COMPLEX ACCORDING TO

$$mM + nA \rightleftharpoons M_mA_n$$

Briefly resuming the theory (for details see Ref.[5]) we shall consider the systems formed by mixing $(1—x)$ volumes of a solution of M ($C_M = C_o$) with x volumes of a solution of A ($C_A = rC_o$), x varying from 0 to 1 (volume changes being neglected). Assuming constant activity coefficients so that the law of mass action is valid in terms of concentrations, the systems, in which the equilibrium $mM + nA \rightleftharpoons M_mA_n$ is supposed to be established, are governed by the equations:

$$K \cdot [M]^m \cdot [A]^n = [M_mA_n];$$
$$C_o(1—x) = [M] + m[M_mA_n];$$
$$rC_ox = [A] + n[M_mA_n];$$

where K denotes the formation constant of M_mA_n. Differentiation of these with respect to x, setting $\frac{d[M_mA_n]}{dx}$ equal to zero and elimination of [M], [A] and $[M_mA_n]$ yield:

$$K \cdot C_o^{m+n-1} \cdot r^{n-1} \cdot m^{1-n} \cdot n^{1-m} \cdot ((n+mr)x_{max} - n)^{m+n} = (r-1)^{m+n-1} \cdot (n-(n+m)x_{max}) \quad (1)$$

where x_{max} denotes the value of x which renders $[M_mA_n]$ maximum. The necessary and sufficient condition for x_{max} to be independent of C_o is:

$$((n + mr)x_{max} — n) = (n — (n + m)x_{max}) = 0$$

from which one obtains:

$$x_{max} = \frac{n}{n+m}; \text{ and } C_M = C_A;$$

This means that on mixing equimolar solutions of M and A the concentration of the complex M_mA_n will reach its maximum value at $x_{max} = \frac{n}{n+m}$, while on mixing non-equimolar solutions x_{max} will depend on C_o, r and K. Therefore, if x_{max} can be determined experimentally it is possible

(I) to determine the stoichiometric ratio of M and A in the complex from experiments with equimolar solutions, and

(II) to calculate the value of K from experiments with non-equimolar solutions.

It is to be noticed that from the first type of experiments we do not obtain the values of m and n but only their ratio. Their actual values can be obtained from the second type of experiments by trial and error, since only insertion of correct values in (1) will lead to a constant value of K.

The value of x_{max} can be determined experimentally from measurements of the value, P, of a particular physical property of the solutions if, and only if P is of the form:

$$P = s_1 [M] + s_2 [A] + f([M_mA_n]);$$

where s_1 and s_2 are constants. The procedure is to evaluate the difference, Y, between the measured value of P and the value of P calculated under the

assumption of no complex formation. The function $Y = Y(x)$ will show an extremum at $x = x_{max}$. In order to verify this let us evaluate dY/dx:

$$\frac{dY}{dx} = \frac{d[M_mA_n]}{dx} \cdot \left[\frac{df([M_mA_n])}{d[M_mA_n]} - (s_1m + s_2n)\right]$$

It is seen that for well-behaved functions $f([M_mA_n])$

(I) Y will have an extremum at $x = x_{max}$ (except in the trivial case where $Y = 0$ for all x),

(II) if Y has more extrema than the one at $x = x_{max}$, the x values at which these other extrema are found will vary with C_o in the equimolar experiments.

In particular, if $f([M_mA_n]) = k[M_mA_n]$, Y can have only one extremum.

The assumptions made in the above mathematical treatment are the following:

1) Each of the reactants corresponds to one particular formula, *i. e.*, the reactants do not partake in equilibria (*e. g.* association, protolysis) other than the one in question;

2) The law of mass action is valid in terms of concentrations;

3) The reactants form only one complex.

Assumption No. 1 will often be justified. In case that an equilibrium of the type $A + bB \rightleftharpoons AB_b$ (*e. g.* A being a base taking up protons) is involved, the treatment will still be valid provided special precautions regarding concentrations are taken [7] (in the example chosen pH must be kept constant).

In order to fulfil condition No. 2 the experiments must be carried out at constant ionic strength or better in a constant salt medium of relatively high concentration. This imposes certain restrictions on the choice of property to be measured, excluding for instance such properties as freezing-point depression and other colligative properties, Faraday-effect, conductivity *etc.* rendering the accuracy obtainable by these methods too low. Results of early investigations utilizing such properties and neglecting the fulfilment of condition No. 2 must be considered unreliable (experimental proof of this has been given recently [8]). Recent investigations for the most part have been based on measurements of optical density, this being generally considered the most — and probably the only — satisfactory property. Exceptions are Refs.[9,10].

By far the most important limitation is introduced by assumption No. 3, especially since cases where only one complex is formed seem to be rare exceptions *. Failure to meet this requirement is the reason why most of the complexity constants determined by c.v. must be considered erroneous. In order to detect whether the assumption of only one complex is justified in a particular system Job [5] recommended the application of c.v. to the system utilizing the largest possible intervals of C_o and r. It is obvious that the formation of several complexes generally will influence the experimental values of x_{max} in such a way that the calculations will not lead to a constant value of K. If a constant value of K is obtained this therefore according to Job can be taken as a criterion for the justification of the assumption. An example will show how

* This and the following discussion do, of course, not apply to systems in which the highest complex formed is MA. In such cases the application of c.v. has sometimes proved successful [11].

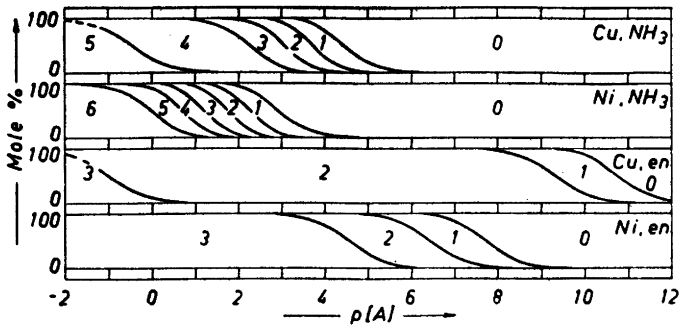

Fig. 1. Ranges of existence for the systems Cu++, NH_3; Ni++, NH_3; Cu++, en; and Ni++, en.

The constants for Cu++, en have been taken from Bjerrum and Nielsen[24], the others from Bjerrum [12]. The symbols 0,1,...6 designate the species M,MA...MA_6. The abscissa is p[A] = −log [A] and for concentrations higher than ~ 1 *M* an activity function of the free amine (see Refs.[12, 24]).

this procedure in some cases has led to erroneous conclusions due to the fact that the largest experimentally available intervals in C_o and r were not sufficiently large or were not employed.

Job [5] applied c.v. to the system cupric nitrate-ammonia measuring the optical density of the mixtures at 615 mμ. Experiments with equimolar solutions were carried out at C_o values between 0.05 and 0.20 mole/l, yielding $x_{max} = 0.80$. Consideration of the ranges of existence for the consecutive cupric ammine complexes (Fig. 1) indicates that in the equimolar experiments almost the total content of cupric ions was employed in the complex $Cu(NH_3)_4^{++}$ at $x = 0.81$—0.82 this complex having a concentration of 0.18—0.19 C_o, while for instance at $x = 0.50$ the cupric ions were distributed approximately as follows: 0.12 C_o Cu^{++}, 0.25 C_o $Cu(NH_3)^{++}$ and 0.12 C_o $Cu(NH_3)_2^{++}$, the total concentration of complexes being approximately 0.37 C_o. Since the lower complexes have considerably smaller extinction coefficients at 615 mμ than the cupric tetrammine ion, it is to be expected that the maximum in *Y* will occur close to $x = 0.80$ indicating the formation of this complex only.

If lower values of C_o had been used, deviations would have been found. Performing experiments at $C_o = 0.00102$ mole/l in order to check this, the present author has found a little pronounced maximum in the *Y* curve at $x = 0.67$ (see Fig. 2, curve I).

In the experiments with non-equimolar solutions Job varied C_{NH_3} between 0.2 and 1.0 mole/l and r between 5.4 and 102. Under these circumstances the position of the maximum in *Y* will essentially be determined by the formation of the cupric tetrammine ion yielding satisfactorily constant, but quite erroneous values of *K* (log $K = 9.3$, correct value log $K = 13.4$, extrapolated from data in Refs.[12, 13]).

If values of r smaller than 4 and low values of C_o had been employed, deviations would certainly have been found. Fig. 2 shows two experiments by the present author. Curve II represents measurements in which $C_o = 0.0102$

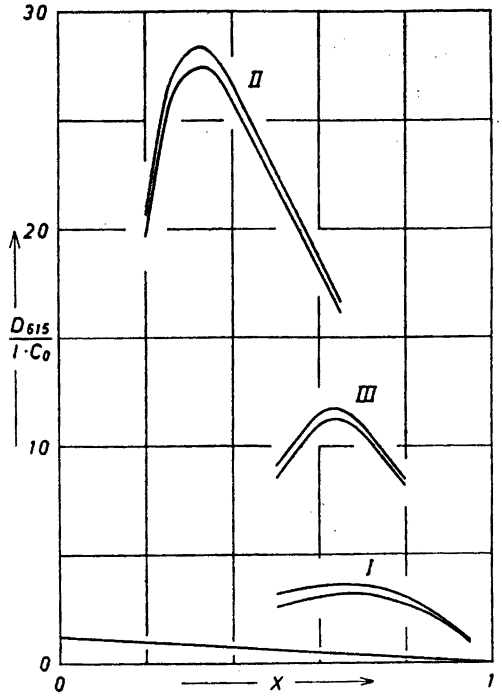

Fig. 2. Continuous variation experiments on the system Cu++, NH_3 in 2 M NH_4NO_3.

The ordinate is the optical density of the solutions at 615 mμ divided by the cell length, *l*, in cm and by the molar concentration of the Cu++ stock solution, C_o. The straight line represents the extinction due to the cupric ions under the assumption of no complex formation. In each pair of curves the upper curve connects the experimental points while the lower curve is the *Y*-curve.

	C_o	r	*l*	x_{max}
I	0.00102	1	10	0.67
II	0.0102	10.3	2	0.33
III	0.0102	2	5	0.64

The measurements were carried out by means of a Beckman DU spectrophotometer with photomultiplier attachment which was kindly put at my disposal by Professor M. Kilpatrick at Illinois Institute of Technology.

mole/l, r = 10.3, whereby $x_{max} = 0.33$ and log $K = 9.89$ which is still in fairly good agreement with Job's value. When decreasing values of r are employed the maximum of the curve moves towards higher x values and should, if the assumption of only one complex were correct, reach $x = 0.8$ at r = 1. However, at $C_o = 0.01$ mole/l the lower complexes become more important so that the maximum is found at an increasing distance to the left of the expected position as r → 1. As a result of this the term $((n+mr)x_{max} - n)$ in (1) decreases so as to reach zero at a value of r greater than one and then becomes negative.

Curve III represents measurements at $C_o = 0.0102$ mole/l, $r = 2$ whereby $x_{max} = 0.64$ which renders the meaningless value of $K = -8.8 \cdot 10^{10}$.

While thus the procedure actually can give the information that the assumption of one complex only is not justified in this case, it is seen that the variations in C_o and r employed by Job were not sufficiently large to reveal this. Similar criticism may be directed towards several c.v. investigations, *e. g.* Refs.[14, 17].

The uncertainty always present as to whether condition No. 3 is fulfilled is a very serious weakness of this application of c.v. which renders the results so obtained quite inconclusive. Different methods for the calculation of the complexity constant have been suggested [2, 6, 10, 11, 18]. None of these, however, remedies the difficulty elucidated in the above example.

FORMATION OF CONSECUTIVE COMPLEXES ACCORDING TO

$$M + nA \rightleftharpoons MA_n; \qquad MA_n + pA \rightleftharpoons MA_{n+p}; \qquad MA_{n+p} + qA \rightleftharpoons MA_{n+p+q};$$

C.v. was first applied to cases of this kind by Vosburgh and Cooper [1], and the most general treatment has been given by Katzin and Gebert [19] who confining themselves to the measurements of optical densities (or any other property depending linearly on the concentrations of M and the complexes) derived the conditional equation:

$$\frac{x_{max}}{1-x_{max}} = n + \frac{p[(e_2-e_o)c_2 + (e_3-e_o)c_3] + q(e_3-e_o)c_3}{(e_1-e_o)c_1 + (e_2-e_o)c_2 + (e_3-e_o)c_3} \cdot \left[1 + \frac{n(c_1+c_2+c_3) + p(c_2+c_3) + qc_3}{C_o(1-x_{max})}\right] - \frac{p(p+n)(c_2+c_3) + q(q+n+2p)c_3}{C_o(1-x_{max})} \qquad (2)$$

in which x_{max} indicates such values of x for which the Y function has extrema, $e_o, \ldots e_3$ indicate the molar extinction coefficients of M and the complexes MA_n, MA_{n+p} and MA_{n+p+q}, and c_1, c_2, c_3 the molar concentrations of the same complexes, while C_o is the concentration of the equimolar solutions of M and A being mixed.

From (2) the conditions for the x values rendering c_1, c_2 and c_3 maximum are obtained by inserting $e_1 \neq e_o$, $e_2 = e_3 = e_o$ and so on as follows:

$$\frac{x}{1-x} = n - \frac{p(p+n)(c_2+c_3) + q(q+n+2p)c_3}{C_o(1-x)} \qquad (3)$$

$$\frac{x}{1-x} = (n+p) + \frac{npc_1 - q(n+p+q)c_3}{C_o(1-x)} \qquad (4)$$

$$\frac{x}{1-x} = (n+p+q) + \frac{n(p+q)c_1 + q(n+p)c_2}{C_o(1-x)} \qquad (5)$$

(3) and (4) being identical with those derived by Vosburgh and Cooper [1] for the case of two complexes only (insertion of $c_3 = 0$):

$$\frac{x}{1-x} = n - \frac{p(p+n)c_2}{C_o(1-x)}; \qquad \frac{x}{1-x} = (n+p) + \frac{npc_1}{C_o(1-x)}$$

These equations make possible an approximate calculation of n and p in cases where the values of the consecutive complexity constants warrant the assumption that c_1 is small when c_2 is maximum and vice versa, provided the corresponding x values can be determined experimentally.

On differentiation of the Y function for the case of two complexes:

$$Y = e_o c_o + e_1 c_1 + e_2 c_2 - C_o(1-x)e_o$$

and of the equation

$$c_o = C_o(1-x) - c_1 - c_2$$

with respect to x and combination of the results one obtains

$$\frac{dY}{dx} = (e_1 - e_o)\frac{dc_1}{dx} + (e_2 - e_o)\frac{dc_2}{dx} \qquad (6)$$

Depending on the sign of the two parentheses, Y may have one or two extrema the positions of which generally bear no simple relation to the composition of the complexes.

However, by a suitable choice of the wavelengths at which the measurements are carried out, it becomes possible in some cases to obtain valuable information.

a) If the wavelength can be so chosen that one of the parentheses in (6) vanishes (*e. g.*, $e_2 = e_o$, $e_1 \neq e_o$) Y will assume its extremum value at the x value which renders one of the complex concentrations maximum (in the example mentioned c_1).

b) If $e_1 = e_2$ the extremum condition for Y reduces to

$$\frac{dc_1}{dx} + \frac{dc_2}{dx} = 0$$

Provided the consecutive complexity constants have sufficiently high values this will take place at an x value close to that at which $\frac{dc_1}{dx} = 0$ ($\frac{dc_2}{dx}$ still being small in this range).

c) If $e_2 \gg e_1 > e_o$ and the consecutive constants are high, Y will continue to increase after c_1 has reached its maximum. Letting Y_1 denote the difference between the experimental value of P and the value calculated under the assumption that all M is converted into MA_n at $x/(1-x) = n$ and that MA_n does not react further with A, and keeping in mind that the high values of the consecutive constants mean that c_o will be vanishingly small when c_2 assumes considerable values, we obtain the following maximum condition for Y_1:

$$(e_2 - e_1)\frac{dc_2}{dx} = 0$$

Y_1 consequently will have its maximum at the x value that renders c_2 maximum.

In cases where more complexes are formed a discussion similar to the above can be carried out, still under the assumption that the consecutive

constants are high enough so that it is possible to divide the Y curve into intervals in each of which one or more of the concentrations may be neglected.

The choice of wavelengths in accordance with the above requirements is usually made in the way suggested by Vosburgh and Cooper, namely as follows. Solutions containing M and A in ratios such as $C_M/C_A = 1:1, 1:2, \ldots, 1:6$ are made up and their spectra recorded. It is assumed that these spectra then constitute close enough approximations to the spectra of the complexes of the corresponding composition as to permit the determination of wavelengths fulfilling conditions of the types mentioned in a), b) and c). A somewhat different approach has been employed by Underwood, Toribara and Neuman [20] but the fundamental assumption is the same.

The usefulness of c.v. in this form has been very differently estimated by different authors [7, 8, 21-24]. We shall undertake a closer examination of the general validity of the assumptions involved.

Let us first direct our attention towards the assumption that the consecutive constants are of sufficiently high values to make the above treatment valid. This assumption is also involved in the methods by which suitable wavelengths are chosen. Before attacking this problem it is appropriate to stress a fact that has mainly been emphasized by N. Bjerrum [25] and J. Bjerrum [12], namely that the distribution of the central ion on the different complexes in a given solution is determined solely by the free ligand concentration. In accordance with this conception the conditions in the solutions under consideration can be very conveniently represented by graphs similar to those in Fig. 1.

Considering such graphs we observe two important facts. In the first place, the initial concentrations of the solutions employed should be high enough to keep the complex under consideration at a high degree of formation. For example, in a solution in which $C_{Ni^{++}}=0.5$ and $C_{NH_3} = 3.0$ the Ni^{++} will not for the most part be present as the 1:6 complex but will be distributed approximately as follows: 50 % $Ni(NH_3)_5^{++}$, 25 % $Ni(NH_3)_6^{++}$ and 25 % $Ni(NH_3)_4^{++}$. At least four times as concentrated solutions must be used in order to make the 1:6 complex prevail. In picking wavelengths it would therefore be wise to examine whether the spectra recorded for the 1:1, 1:2 ... solutions vary considerably with the total concentration. Such examinations have in general not been published in c.v. investigations.

We observe further that in order to justify the above treatment the ranges of existence must be wide enough (the consecutive constants high enough) so that the most part of the central ion can be present in the form of the particular complex under consideration at some appropriate value of the free ligand concentration. As is seen from the graphs this is not always the case. In fact, very often not more than 50 % of the content of central ion can be present in the form of one particular complex at any value of the free ligand concentration. In the case of bi- and poly-dentate ligands the assumption is frequently valid, but in the investigation of unknown systems it is obviously not safe to make predictions regarding these conditions.

In view of these observations it is readily explained why c.v. in many cases has rendered incomplete results. For if the assumptions are not correct, c.v. plots will in general still show maxima, but the interpretation of such data

is apt to lead to unreliable results. An example is the investigation of the cupric-ammonia system by Vosburgh and Cooper [1], to which both of the above objections are valid.

In some cases difficulties of a different nature may arise. The mathematical treatment remains valid if M or A partake in protolytic equilibria provided pH is held constant (see p. 301). It breaks down, however, if polymerizations are involved. Not only will the x values rendering the Y functions maximum be displaced, but if the polymerization products are coloured this may further influence the results. Caution is therefore necessary in cases such as for instance the one referred to by Martell and Calvin [3] as simple and clear-cut, namely the investigation of the uranyl-sulfosalicylate system by Foley and Anderson [26], in which polymerization and hydrolysis of the uranyl ion are likely to take place at the higher pH values.

In recent work [19,27,35] Katzin and co-workers have utilized the light absorption in the ultraviolet of some ligands (NO_3^-, SCN^-) in c.v. experiments. While Beer's law usually holds within the experimental error for transition metal ions in aqueous solution, the optical density of for instance nitrate solutions is not proportional to the formal nitrate concentration. It appears reasonable to expect that the formation of ion pairs or similar aggregates of ions in which the distance between the ions is small will influence the spectra of the ions involved, and more so the less solvated and the more polarizable the ions. In organic solvents of low dielectric constant such interaction is likely to take place to a higher degree than in water. The spectral behaviour of the colourless anions in aqueous and organic solutions is not very thoroughly investigated but at least the spectrum of the nitrate ion appears to be dependent to a considerable degree on the surroundings. Such phenomena may seriously affect the validity of the assumptions involved in c.v., and it is suggested that the validity of Beer's law for the species in question be tested whenever c.v. is extended to fields where this is not already well established.

COMPARISON BETWEEN C. V. AND J. BJERRUM'S METHOD OF CORRESPONDING SOLUTIONS

Finally attention should be called to the fact that another purely optical method of similar experimental simplicity is available for the solution of problems of the type to which c.v. is generally applied, namely J. Bjerrum's method of corresponding solutions [28]. This method may be applied to systems in which complexes of the type MA_n are formed by reversible step reactions, the only condition for the application being that the spectra of the species M, MA ... MA_n are different.

Two solutions (referred to by subscripts 1 and 2) of different total concentrations of M and A are said to be corresponding if:

$$\bar{n}_1 = \bar{n}_2, \text{ and therefore } [A_1] = [A_2],$$

where $\bar{n} = \frac{C_A - [A]}{C_M}$ is the formation function. Procedures for finding corresponding solutions have been devised [28]. Since C_{A1}, C_{A2}, C_{M1} and C_{M2} are known it is possible to calculate [A] and n so that each pair of corresponding

solutions yields one point of the formation curve. When the formation curve has been determined the complexity constants may be calculated by standard procedures [30] assuming that all the steps MA ... MA_{n-1} are represented in the equilibrium. This assumption seems to be of a very general if not universal validity. The fact that the formation curves for some systems may be adequately described in terms of fewer parameters than all of the consecutive complexity constants as pointed out by Wormser [29] does not make the logically more satisfactory and in some cases clearly proven conception of a stepwise formation less probable. If in a particular system some of the steps are not employed this will find its expression in the values of the corresponding constants.

The method of corresponding solutions has been applied to the cupric-ammonia system [28] in which case the results were in agreement with those obtained by other methods, to the cupric-chloride system [31] and to the stepwise formation of aquo metallic ions in alcoholic solution [32]. Olerup has independently applied a similar principle to the ferric-chloride system [33].

The method of the corresponding solutions has two advantages over the method of continuous variations:

(I) When applicable, it furnishes quantitative results in terms of complexity constants.

(II) When not applicable, *i. e.*, when the complexity constants or the extinction coefficients have values which make the experimental accuracy low, this is clearly indicated by the experimental results.

The failure of c.v. in this respect makes it an indicative rather than a conclusive method.

Acknowledgment. I wish to express my gratitude to professor J. Bjerrum for having suggested this investigation and for his kind encouragement and criticism during the work.

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Enzymatic Hydrolysis of Organophosphorus Compounds

V. Effect of Phosphorylphosphatase on the Inactivation of Cholinesterases by Organophosphorus Compounds *in vitro*

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The effect of phosphorylphosphatase of human serum on the inactivation of cholinesterases by organophosphorus compounds *in vitro* was studied.

The esterase inactivation is a progressive reaction. Phosphorylphosphatase cannot reactivate inactivated cholinesterases when the former is added after maximum esterase inactivation is reached. On the other hand, phosphorylphosphatase stops the inactivation at a certain level when added before maximum inactivation is reached.

The results are discussed from a kinetic point of view.

The existence of enzymes, phosphorylphosphatases, catalysing the hydrolytic breakdown of organophosphorus compounds has been reported recently [1,2]. A series of previous papers discussed some properties of the phosphorylphosphatase ("tabunase") of human blood serum [3], the effect of reversible cholinesterase inhibitors on that enzyme [4], and the enzyme specificity [5]. In the present paper the effects were studied when a phosphorylphosphatase preparation was added to cholinesterases, inactivated by various organophosphorus compounds.

METHODS AND MATERIAL

Phosphorylphosphatase and cholinesterase activities were measured by the Warburg technique [1,6]. Fraction IV-1 of human postpartum serum was used as enzyme source for phosphorylphosphatase. Acetylcholinesterase (AChE) was a partly purified preparation of the electric tissue of *Torpedo marmorata*, and Fraction IV-6 of human serum was used as a (butyryl-) cholinesterase (ChE) preparation.

The following organophosphorus compounds were used: tabun (dimethylamidoethoxy-phosphoryl cyanide), DFP (di*iso*propoxyphosphoryl fluoride), TEPP (tetraethylpyrophosphate), and mintacol (E 600, diethyl-*p*-nitrophenyl-phosphate). Acetylcholine chloride was employed as substrate for cholinesterases; its concentration during the esterase determination was usually 1.10×10^{-2} *M* (total volume of the reaction mixture, 2.00 ml).

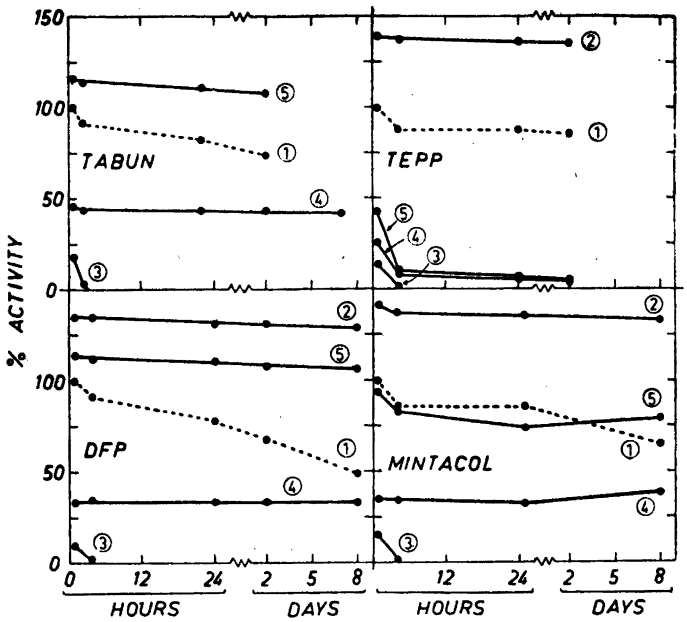

Fig. 1. Effect of phosphorylphosphatase (Fraction IV-1) on acetylcholinesterase (AChE, electric tissue) and on the inhibition of this esterase by various organophosphorus compounds (P) tested after various periods of time. Esterase activity tested against acetylcholine (1.10 × 10^{-2} M). Total volume of the incubation mixture, 1.6 ml, kept at 4° C. Esterase and phosphatase respectively were incubated 15 minutes with the organoposhphorus compound prior to further treatment. Concentrations of the organophosphorus compounds during incubation: tabun, 4.65 × 10^{-6} M; DFP, 1.17 × 10^{-3} M; TEPP, 1.58 × 10^{-5} M; mintacol, 7.42 × 10^{-5} M. 1) AChE; N.B. the phosphatase preparation has an esterase activity which is appr. 9 % of that of AChE; 2) AChE + phosphatase; 3) AChE + P; 4) (AChE + P) + phosphatase; 5) (Phosphatase + P) + AChE.

RESULTS

The effect of phosphorylphosphatase on inactivated cholinesterases. Acetylcholinesterase (AChE) and cholinesterase (ChE) were incubated for 15 minutes with an organophosphorus compound (P). Phosphorylphosphatase was then added and the first measurement of the esterase activity was performed after a further period of 45 minutes (Figs. 1 and 2, No. 4). In another series the phosphorus compound was incubated for 15 minutes with phosphorylphosphatase; then the cholinesterase was added and the activity of the latter determined (No. 5). The concentration of the phosphorus compound in each case was chosen to give 95 % or more inactivation of cholinesterases when the esterase was incubated for 45 or 60 minutes prior to the addition of substrate (acetylcholine) (No. 3). In a control series, the effect of phosphorylphosphatase on the cholinesterase activity was determined (Nos. 1 and 2). All five reaction mixtures were kept at 4° C and the cholinesterase activities tested after various periods of time. The results thus obtained with tabun, DFP, TEPP and mintacol are recorded in Fig. 1 (AChE) and Fig. 2 (ChE).

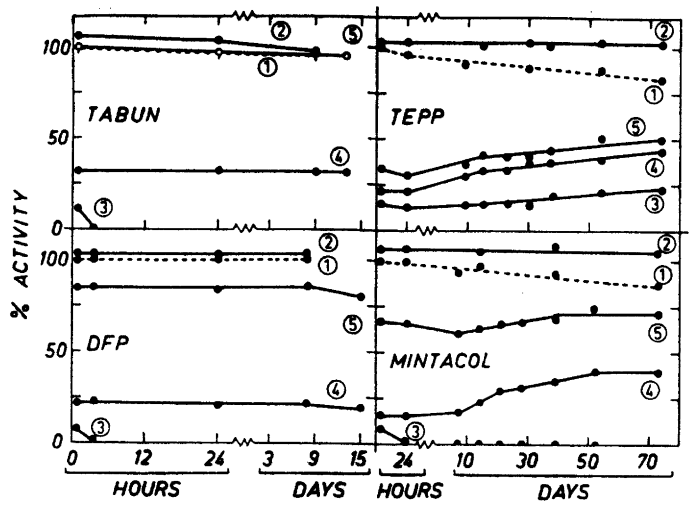

Fig. 2. Effect of phosphorylphosphatase (Fraction IV-1) on serum cholinesterase (Fraction IV-6) and on the inhibition of this esterase by various organophosphorus compounds (P) tested after various periods of time. Concentrations of the organophosphorus compounds during incubation: tabun, 2.33 × 10^{-6} M; DFP, 9.36 × 10^{-7} M; TEPP, 8.44 × 10^{-7} M; mintacol, 4.45 × 10^{-6} M. Numbers and further explanations as in Fig. 1.

It will be noted that the phosphatase preparation (Fraction IV—1) has a weak cholinesterase activity not exceeding 10 % of the activity of the cholinesterase preparation used.

Acetylcholinesterase (Fig. 1). A characteristic property of the phosphatase preparation is that it activates the acetylcholinesterase activity but not the (butyryl)cholinesterase activity (*cf.* Fig. 2). This activation (curve No. 2) will be discussed in a forthcoming publication. As was expected from the results described earlier —, the esterase inhibiting effect of tabun is lost by incubation with phosphatase (No. 5). This is also true for DFP. In both these cases the activating effect of the phosphatase preparation is partly retained even after incubation with the phosphorus compound. The latter effect, however, is lost when phosphatase hydrolysed mintacol. The situation is completely different for TEPP. We know that TEPP is enzymatically hydrolysed by the phosphatase preparation, but contrary to the other compounds tested the degradation products of TEPP seem to have a strong esterase inhibiting effect (No. 5).

The effect of phosphatase on inactivated acetylcholinesterase is seen in curve No. 4. After 15 minutes incubation with tabun, DFP and mintacol, the addition of phosphatase stops the esterase inactivation at a certain level, but there is no reactivation of the esterase activity even after 15 days (not indicated in Fig. 1) of incubation with phosphatase. TEPP seems to inactivate acetylcholinesterase to a maximum more rapidly (in 15 minutes or less) than do the other compounds tested. We conclude from these results that phosphatase stops the progressive irreversible inactivation of acetylcholinesterase

when added before the maximum effect of the inactivator is reached. Phosphatase, however, is not capable of reactivating the inactivated acetylcholinesterase under the experimental conditions used.

The effect of phosphatase on the process of acetylcholinesterase inactivation has been studied further and is discussed below.

Cholinesterase (Fig. 2). Phosphatase has no activating effect on human serum cholinesterase: there is a small increase of cholinesterase activity in the presence of phosphatase, but this effect is due to the weak esterase activity of the phosphatase preparation itself. A great part of the cholinesterase inactivating effect of phosphorus compounds is lost after incubation with phosphatase. The reaction products from this hydrolytic procedure, however, are still effective as inactivators of cholinesterase; this is contrary to the results obtained with acetylcholinesterase.

Phosphatase has no reactivating effect on cholinesterase inactivated by tabun or DFP (No. 4). For mintacol a slow but significant reactivation was observed. This reactivation must be catalysed by the phosphatase present, since a control experiment in the absence of phosphatase did not show this phenomenon. For TEPP, however, a spontaneous reactivation by hydrolysis is known to occur from previous observations [7,8]; phosphatase seems to catalyse this reactivation procedure (No. 4) to a small extent during the first days. From these experiments we conclude that phosphatase has no (as far as tabun and DFP are concerned) or a very weak reactivating effect on inactivated cholinesterase. However, as in the case of acetylcholinesterase, phosphatase stops the inactivation of cholinesterase when it is added before maximum inactivation is reached.

The progressive nature of the cholinesterase inactivation by tabun. The reaction rate at which cholinesterases are inactivated by an organophosphorus compound is different for various compounds and is also dependent on the nature of the enzyme preparation and on the concentration of the phosphorus compound used. This progressive nature of cholinesterase inactivation by tabun is illustrated in Fig. 3. Acetylcholinesterase was incubated with tabun and the esterase activity was then measured after 0, 10 and 20 minutes by adding acetylcholine to the incubation mixture (Fig. 3, A). The concentration of tabun used was such that after 60 minutes incubation of the esterase, the activity of the latter was reduced 95 %. It is seen in Fig. 3 that after 10 minutes incubation the esterase activity is reduced to 58.5 % and after 20 minutes to 39 %.

In a previous paper [9] it was demonstrated that acetylcholinesterase is protected by acetylcholine against the inactivation by tabun if the acetylcholinesterase is added before or at the same time as the inactivator. In Fig. 3 (B) the results are shown when acetylcholine is present from zero time and tabun is added to the enzyme-substrate mixture 10 and 20 minutes afterwards. The protective effect of the substrate against esterase inactivation by tabun is significantly demonstrated in this experiment.

The effect of phosphorylphosphatase on the process of cholinesterase inactivation. In a preliminary experiment, performed with acetylcholinesterase, and somewhat different from those described above, the effect of phosphorylphosphatase on the tabun-inactivated esterase was studied (Table 1) by simul-

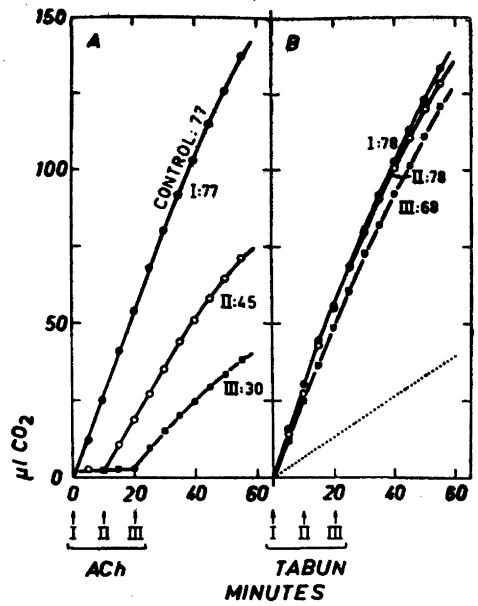

Fig. 3. The progressive nature of acetylcholinesterase inactivation by tabun. A) Esterase incubated with tabun from zero time; acetylcholine (ACh) added simultaneously with tabun (I), and after 10 (II) and 20 (III) minutes incubation. Arabic numbers refer to esterase activity (in b_{30} values). B) Acetylcholine present from zero time and tabun added simultaneously with ACh (I) and after 10 (II) and 20 (III) minutes.

taneous addition to the esterase of the phosphatase preparation and the acetylcholine (substrate). It was found that the activating effect of the phosphatase preparation on the esterase activity is not influenced by tabun. Furthermore, the low cholinesterase activity of the phosphatase preparation is reduced by

Table 1. Effect of phosphorylphosphatase (Fraction IV-1) on the inactivation of acetylcholinesterase (AChE, electric tissue) by tabun. AChE was incubated 45 minutes with tabun before the substrate (acetylcholine) or substrate + phosphatase was added. Phosphatase was added to AChE at the same time as the substrate, except in the last experiment where tabun and phosphatase were together 45 minutes before acetylcholine was added. Reaction mixture, 2.00 ml. In the two experiments (I and II) the concentration of tabun was different (appr. 2×10^{-8} M).

Enzyme system	Exp. I	Exp. II
AChE	91.5	154
AChE + Phosphatase	108.5	167
AChE + Tabun	20	76.5
(AChE + Tabun) + Phosphatase	31	89.5
Tabun + Phosphatase	8	10.5
(Tabun + Phosphatase)	1.5	6

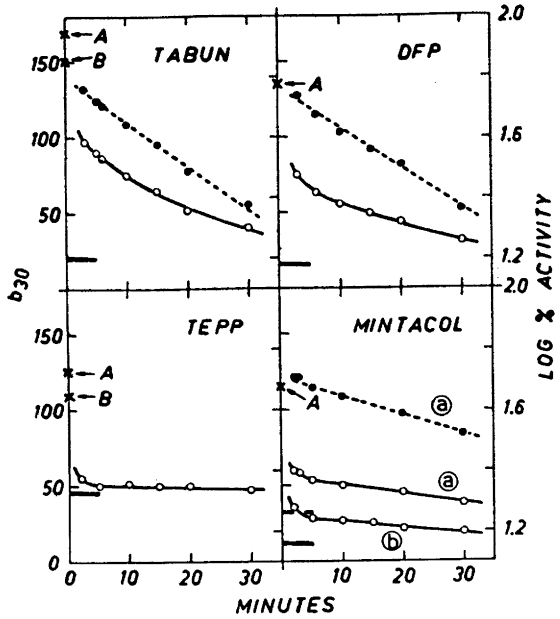

Fig. 4. Inactivation of acetylcholinesterase (AChE, electric tissue) by organophosphorus compounds. The reaction was stopped by adding phosphorylphosphatase (Fraction IV-1) after various periods of time. Esterase activity expressed in b_{30} values (left ordinate and full drawn lines). The logarithm of per cent activity (right ordinate and dotted lines) was calculated with the activity of the mixture AChE + phosphatase as control. Short and wide lines (on the ordinates) give the activity at maximum inactivation. A: activity of the AChE-phosphatase mixture; B: activity of the AChE preparation alone. Concentrations of the organopshophorus compounds: tabun, 4.16 × 10^{-8} M; DFP, 3.63 × 10^{-6} M; TEPP, 2.56 × 10^{-8} M; mintacol, a) 2.16 × 10^{-7} M; b) 4.32 × 10^{-7} M.

tabun. In the complex system, consisting of acetylcholinesterase + phosphatase + tabun + acetylcholine, we have to consider these two facts, *i. e.*, the activating effect on the esterase by phosphatase not influenced by tabun, and the inhibiting effect of tabun on the esterase activity of both acetylcholinesterase and the low one of the phosphatase preparation.

We have learned from the experiments described above that the inactivation of cholinesterases in most cases is progressive and it is surmised that it proceeds as a stepwise reaction (see further "Discussion" below). The effect of a phosphorylphosphatase preparation on the process of inactivation was therefore studied in more detail, especially the effects obtained by a certain concentration of the phosphorus compound before maximum inactivation was reached. The results of these experiments are shown in Figs. 4 and 5 and were obtained by employing the following experimental conditions. The phosphorus compound was added to the esterase and after various short periods of time (2 to 30 minutes) a phosphorylphosphatase preparation (Fraction IV—1) was added. The cholinesterase activity of that mixture was then determined with

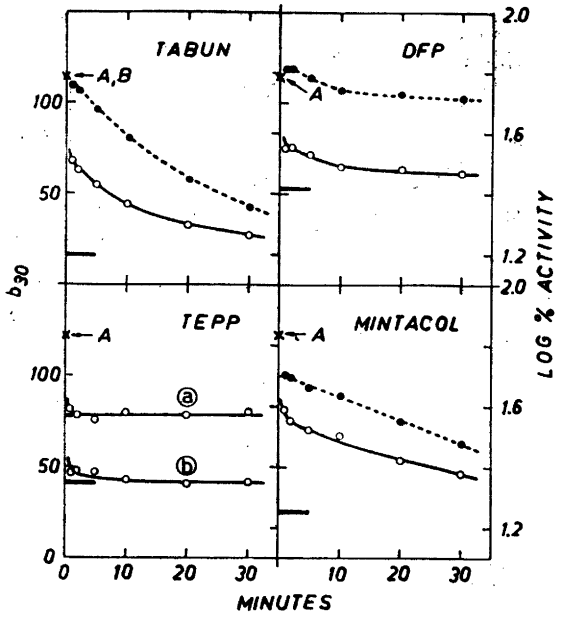

Fig. 5. Inactivation of cholinesterase (Fraction IV-6 of human serum) by organophosphorus compounds. See Fig. 4 for further explanations. Concentrations of the organophosphorus compounds: tabun, 1.66 × 10^{-5} M; DFP, 2.90 × 10^{-5} M; TEPP, a) 1.53 × 10^{-5} M; b) 3.07 × 10^{-5} M; mintacol, 1.15 × 10^{-5} M.

acetylcholine as substrate. It will be remembered (see Figs. 1 and 2) that the phosphatase has no effect on the esterase which beforehand was inactivated maximally by a specific concentration of a phosphorus compound.

For both acetylcholinesterase (Fig. 4) and serum cholinesterase (Fig. 5) the progressive enzyme inactivation by tabun, DFP and mintacol can be stopped by adding phosphorylphosphatase. Once the phosphatase is added there is no further decrease of esterase activity. It has been shown that all of the inactivator added reacts immediately with the esterase and this reaction can be reversed by phosphatase. In a second irreversible reaction the esterase is inactivated and this reaction is not influenced by the phosphatase. TEPP differs from the other three compounds studied in the very rapid reaching of maximum inactivation of the esterases. Even after one minute incubation with TEPP maximum inactivation is reached and therefore the addition of phosphatase after that period has no influence on esterase activity.

DISCUSSION

The mechanism of enzyme inhibition by organophosphorus compounds has been discussed in a variety of publications during the last few years. These compounds seem to be general inhibitors for enzymes having carboxylic esterase activity, *e. g.*, chymotrypsin, trypsin, liver esterase, acetylesterase, B-

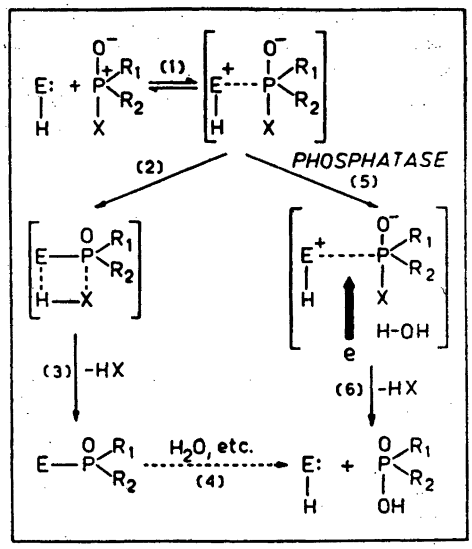

Fig. 6. Tentative scheme of the reaction mechanism for the inactivation of cholinesterases (E) by organophosphorus compounds, and the effect of phosphorylphosphatase (e) on that reaction.

esterase [10]. The inhibitory process for all these enzymes is a first order reaction and bimolecular. It has been demonstrated for some of these enzymes that the inhibitor phosphorylates the enzyme [11]. As far as cholinesterases are concerned most studies are consistent with the view that a similar reaction mechanism is valid for the inactivation of these enzymes by organophosphorus compounds. The analogy between this phosphorylating process and the reaction mechanism for the hydrolysis of acetylcholine by cholinesterases has been pointed out by Wilson *et al.*[12]

The reaction mechanism of the spontaneous hydrolysis of organophosphorus compounds was discussed by Larsson [13] and the similarity between the base-catalysed hydrolysis and the enzymic hydrolysis of these compounds was postulated by the present authors [3]. In some preliminary experiments carried out to study the effect of phosphorylphosphatase on phosphorylated cholinesterases the results were leading to the idea that the phosphatase is able to reactivate inactivated cholinesterases [14]. A more detailed study of this phenomenon, as made in the present paper, showed that there is no true enzymic reactivating effect, but that the phosphatase can stop the progressive inactivation when added before maximum inactivation is reached.

On the basis of investigations by Jansen, Aldridge, Wilson, Wagner-Jauregg, Myers, and others, and those from this laboratory, the following reaction mechanism is suggested (Fig. 6). The enzyme (E) combines reversibly (1) with the phosphorus compound by a one-point contact at the ester-bonding site (*i. e.*, the "esteric" site [7] or active group II [8] of the acetylcholinesterase molecule); then the acid group (X) of the phosphorus containing part (CN in

tabun) of the complex reacts (2, 3) with a hydrogen atom of the enzyme molecule releasing the corresponding acid. The dephosphorylating reaction (4) of the inhibited enzyme is, for most phosphorus compounds, very slow compared with reactions (1), (2) and (3), and therefore the enzyme remains inhibited (inactivated). Catalysis of reaction (4) by replacing water with hydroxylamine or derivatives of hydroxamic acid has recently been demonstrated by Wilson and Meislich [15].

On the basis of the results described in the present paper it is now suggested that phosphorylphosphatase (e) prevents the reactions (2) and (3) from taking place (see reaction 5). The phosphorus containing moiety of the loosely bound complex (reaction 1) is thereby hydrolytically split, acid (HCN in the case of tabun) and a phosphoric acid are released, and the cholinesterase becomes active (6). The degree of the effect of the phosphatase depends both on the time elapsing after cholinesterase and phosphorus compound have come in contact with each other, and on the various groups (alkyl, substituted amido) of the phosphate inhibitor.

The authors wish to express their sincere thanks to Professor Gustaf Ljunggren, Chief of the Research Institute of National Defence, Department 1, for his continual interest in these investigations. We are indebted to Ing. Henrik Björling of A/B Kabi, Stockholm, for the preparation of Fraction IV-1.

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Estimation of Phenolic Hydroxyl Groups in Lignin

I. Periodate Oxidation of Guaiacol Compounds

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A method for the estimation of free phenolic groups in softwood lignin has been developed. The method is based on the finding that the action of sodium periodate upon guaiacol results in rapid oxidative demethylation, methanol being liberated in a yield of about 0.9 mole per mole of guaiacol. A variety of substances containing one or two guaiacyl nuclei and representing the probable structural principles of lignin have been investigated with regard to their behaviour towards periodate. Substituents in the positions *para* and *ortho* to the phenolic hydroxyl group of guaiacol in general were found to be without influence upon the methanol formation. A few exceptions, which, however, are of minor importance to the lignin problem, are pointed out.

As a result of these model experiments, it is concluded that any amount of methanol (moles of methanol per methoxyl group) obtained on periodate oxidation of softwood lignin preparations, when multiplied by 1.1, will give the number of phenolic hydroxyl groups per methoxyl in the lignin preparation examined.

One of the main problems of lignin chemistry is to elucidate the way in which the guaiacyl propane monomers (in softwood lignin) or the guaiacyl and syringyl propane monomers (in hardwood lignin) are linked to each other to form the lignin molecules. Previous work (*cf.*[1]) suggests that different types of linkages are involved, *viz.*,

a) alkyl aryl ether linkages connecting the propane side-chain of one monomer with the aromatic nucleus of the following one. The alkyl carbon atom can be either the α-carbon (type a_1) or possibly the β-carbon (type a_2) of the side-chain;

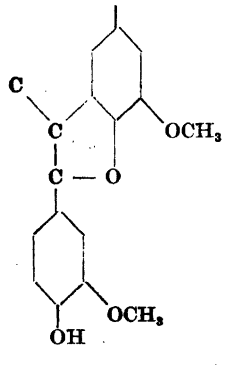
b) carbon-carbon linkages between the side-chain of one monomer and the aromatic nucleus of the following one;

c) alkyl ether linkages between two side-chains;

d) carbon-carbon linkages between two side-chains.

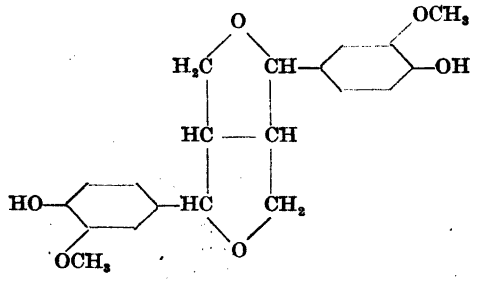
A combination of types a_1 and b is present in the coumaran ring structure (I), whereas a combination of types c and d is to be found in pinoresinol (II). Both structures have been shown to be formed as intermediates in the bio-

synthesis of lignin from coniferyl alcohol, and the coumaran structure at least is assumed to constitute an essential building element in the final lignin molecule [1] (*cf.*, however,[2]). It has been proposed that alkyl ether linkages (c) of the benzyl alkyl ether type are involved in sulphonation, alcoholysis, and other typical reactions of lignin [3,4]. Finally, the occurrence in lignin of alkyl aryl ether linkages of type a_2 (*iso*-propyl aryl ether linkage, III) has been discussed on the basis of model experiments [5,6].*



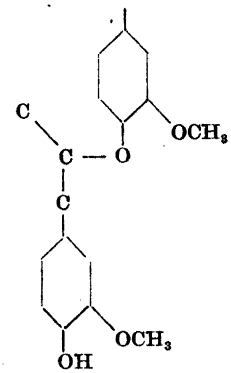
I

(linkages a_1 and b)



II

(linkages c and d)



III

(linkage a_2)

As yet, conclusive information regarding the relative amounts of the various kinds of linkages in lignin is missing. Obviously, for a further elucidation of this problem, a knowledge of the number of free phenolic groups in lignin must be of fundamental interest. If this number were known (*n* phenolic groups per phenylpropane monomer), the number of the alkyl aryl ether bonds (a_1 plus a_2) would also be known (1—*n*).

Moreover, a determination of any change in the number of phenolic groups taking place if lignin is subjected to reactions such as sulphonation and alcoholysis, would be of great value for the understanding of the mechanism of these reactions and the structure of the reacting groups.

The need of correct data concerning the phenol content of lignin was early realized, and a variety of methods for its estimation have been used in the past; they yielded, however, widely differing figures (*cf.* the summaries on lignin [7,8]). Conflicting values have also been reported very recently. Whereas Freudenberg [9,1], using the reaction of phenolic hydroxyl groups with 2,4-dinitrofluorobenzene and other methods, found 0.5—0.6 phenolic hydroxyls per methoxyl group (in lignosulphonic acid), Aulin-Erdtman [10,11], as a result of her extensive spectrophotometric investigations, concludes that lignosulphonic acid contains 0.17—0.25 phenolic groups per methoxyl. Values of 0.23—0.24, likewise obtained spectrographically, have been reported by Goldschmid [12].

* *Note added in proof:* The formation of linkages of type a_2 (III) on enzymatic dehydrogenation of coniferyl alcohol was recently demonstrated by K. Freudenberg, H. Schlüter and W. Eisenhut *Naturwiss.* **41** (1954) 576.

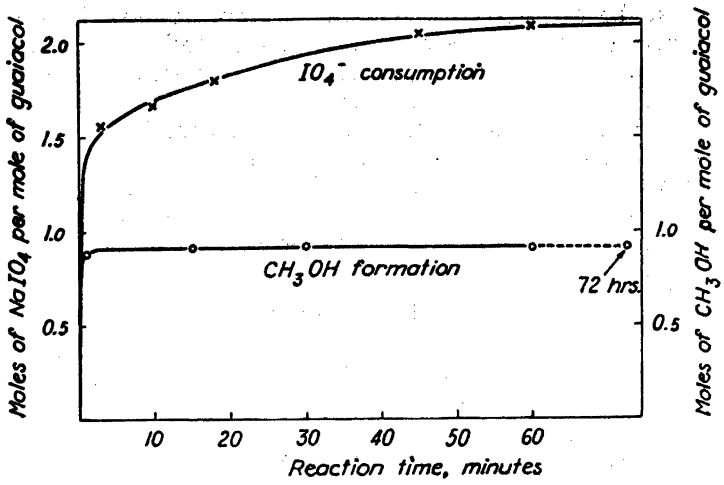


Fig. 1. Consumption of periodate and formation of methanol on periodate oxidation of guaiacol. (0.5 millimole of guaiacol and 3.5 millimoles of sodium metaperiodate in 50 ml of water, at 4°.)

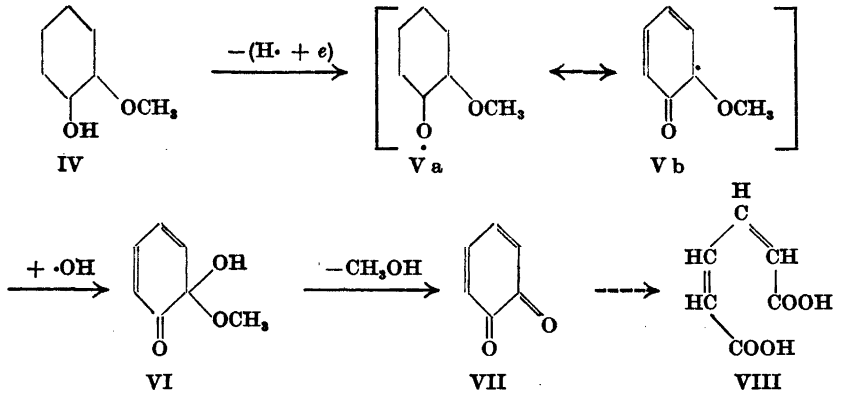
In this laboratory, a new method for the estimation of free phenolic groups in lignin has been developed. The present communication deals with an examination of the method using lignin model substances, and in following papers some results obtained with various lignin preparations will be reported. Preliminarly it may be stated that, with comparable lignin preparations (lignosulphonic acids), our method yielded values similar to those given by Aulin-Erdtman [11] and Goldschmid [12].

The new method is based upon the behaviour of phenols, especially guaiacol and substituted guaiacols, towards periodic acid. Whereas this reagent has been extensively used for many years in the cleavage of C—C linkages in α-glycols, α-ketols and related structures, very little attention has been paid to its action upon phenols. The only work covering the latter problem to be found in the literature is contained in two short communications by Pennington and Ritter [13,14]. These authors found that carbohydrate-free preparations of lignosulphonic acids consumed periodic acid, the products recovered after the oxidation containing less methoxyl than the original material. Methanol was detected in the reaction mixture. It was also shown that various phenols, including guaiacol and some substituted guaiacols such as vanillyl alcohol, vanillic acid, and ferulic acid, were attacked by periodic acid. In the two last-mentioned cases amorphous oxidation products were formed, the methoxyl contents of which were considerably lower than those of the original acids.

In connection with the synthesis of guaiacylglycerol, Adler and Yllner [15] investigated the periodate breakdown of the glycerol side-chain of this substance. As could be expected from the results of Pennington and Ritter, a simultaneous attack upon the guaiacyl nucleus occurred. A comparison with the behaviour of guaiacol revealed that in both cases a very rapid initial consumption of periodic acid, followed by a slower further oxidation, took place.

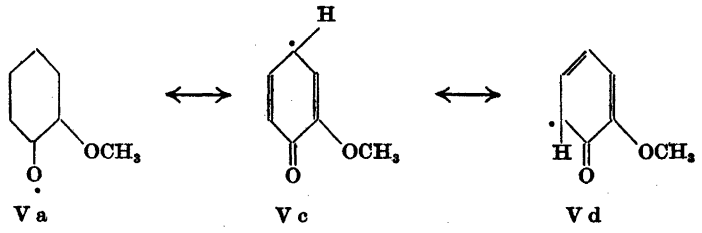
It seemed to be of interest to examine whether the periodate oxidation of these substances involved any formation of methanol, similar to that observed by Pennington and Ritter in the case of lignosulphonic acid. Surprisingly enough, it was found that no less than 90—92 % of the amount of methoxyl groups present were rapidly liberated as methanol, when guaiacol in aqueous solution was treated at 4° C with an excess of sodium periodate. The total amount of methanol was formed during the initial, rapid phase of periodate consumption (Fig. 1). Under the conditions used (4°, dark room), the methanol formed was stable against the excess periodate present during a period of several days (*cf.*[16]).

Regarding the mechanism of the oxidative methanol formation, the following sequence of reactions may be tentatively suggested:



The primary attack of periodate upon guaiacol will result in the formation of the mesomeric radical V. This view regarding the initial step in the oxidation of phenols by various oxidants has been put forward by Pummerer [17] and has been generally accepted (*cf*[18]). Recently, it has received further experimental support by the isolation of the comparatively stable 2,4,6-tri-*tert.*-butylphenoxyl radical [19] from the dehydrogenation of the corresponding phenol with lead dioxide and similar oxidants. In a second oxidation step, the radical V, in its mesomeric form V b, may react with an hydroxyl radical, yielding the hemiacetal VI. This will spontaneously lose one mol. of methanol, thus being converted into *o*-quinone (VII), which finally undergoes periodate oxidation to *cis-cis*-muconic acid (VIII). The last-mentioned product has been isolated from the reaction mixture.

Since the yield of methanol is 90—92 %, the reaction sequence proposed above appears to be the main pathway of the oxidation. Only 8—10 % of the guaiacol used is oxidized in a different way, obviously not including the hypothetical intermediate VI, which is supposed to be the immediate source of the methanol. These side-reactions might be explained by the assumption that the further oxidation of the aroxyl radical, to an extent of 8—10 %, arises from the mesomeric forms c and d:



As has been shown recently by Wessely [20], the action of lead tetraacetate upon guaiacol results in the formation of methoxy-*p*-quinone in a yield of 57 %. This oxidant thus seems to attack mainly the mesomeric form V c.

The scope and the mechanism of the oxidative cleavage of *o*- (and *p*-) hydroxyaryl ethers as well as the reaction of other types of phenols with periodic acid are being further studied in this laboratory.

In contrast with guaiacol, veratrol was found to be completely stable in the presence of sodium periodate under the experimental conditions used. It is interesting to note that, in this respect, periodic acid differs from other specific oxidants, like percarboxylic acids, which have been shown to oxidize not only pyrocatechol [21] — with the formation of muconic acid — but also veratrol [22], with the formation of dimethyl muconate.

These findings seemed to indicate that the determination of methanol formed on periodate oxidation of lignin might offer a possibility of estimating the amount of phenolic guaiacyl residues present in softwood lignin. It was obvious, however, that correct values would not be obtained unless the following conditions were fulfilled:

1) All types of phenolic guaiacyl groups in lignin, irrespective of their way of substitution, yield similar amounts of methanol;

2) Methanol is formed solely from the phenolic nuclei, and not from other structural elements in lignin.

The following experiments constitute an examination of point 1, *i.e.*, a study of the methanol formation from various guaiacol derivatives related to possible structural elements in lignin. The validity of point 2 will be discussed in a subsequent paper.

Table 1 gives the yields of methanol obtained from a series of guaiacol derivatives. The oxidation was carried out with an excess of sodium periodate either in aqueous solution or in 80 % acetic acid. After precipitation of iodate and unconsumed periodate with a lead nitrate solution, methanol was distilled off and determined by oxidation with permanganate and colorimetric measurement of the formaldehyde obtained (*cf.* exptl. part). Several (generally 3 to 6) samples of each substance were oxidized for various periods of time as shown for guaiacol in Fig. 1. The rate of methanol formation was slightly lower in 80 % acetic acid than in water. In the case of guaiacol, for instance, the maximum value was reached after a reaction time of 1—2 minutes in aqueous solution (*cf.* Fig. 1) and after 5—6 minutes in the acetic acid. The final methanol values, however, were practically equal in both types of experiments (*cf.* substances IV, XI, and XXIX, Table 1). The methanol figures given in Table 1 represent the average of the values obtained after various times of reaction, excluding values relating to the rapid initial phase (*cf.* Fig. 1). In some cases the periodate consumption was followed and found to procede in a generally similar way in both solvents, the total periodate consumption in the slower phase, however, being somewhat greater in acetic acid than in water.

The substances in Table 1 have been divided into four groups, A—D.

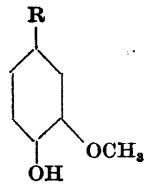
Group A comprises substances in which the guaiacol nucleus is substituted in the 4-position, with no double bond conjugated with the phenolic ring present. In addition to some guaiacyl carbinols (IX—XI, XVI), a guaiacyl

Table 1. Formation of methanol on periodate oxidation of guaiacol derivatives.

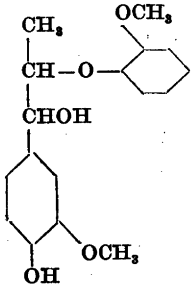
Substance	Method of preparation	Moles of CH_3OH per mole of substance	
		in water	in 80 % acetic acid
Group A:			
Guaiacol (IV)	—	0.91	0.92
Vanillyl alcohol (IX)	23	0.88	—
α-(4-Hydroxy-3-methoxyphenyl)-ethanol (X)	*cf.* exptl. part	0.88	—
α-(4-Hydroxy-3-methoxyphenyl)-*n*-propanol (XI)	24	0.94	0.91
Vanillyl ethyl ether (XII)	*cf.* exptl. part	0.89	—
Barium 1-(4-hydroxy-3-methoxyphenyl)-*n*-propane-1-sulphonate (XIII)	»	0.92	—
Eugenol (XIV)	—	—	0.89
1-(4-Hydroxy-3-methoxyphenyl)-2-propanone (XV)	25	—	0.88
*Iso*eugenolglykol β-guaiacyl ether (XVI)	26	—	0.90
Dehydro-di*iso*eugenol (XVII)	27	—	0.92
Dihydro-dehydro-di*iso*eugenol (XVIII)	28	—	0.93
Dehydro-diconiferyl alcohol (XIX)	29	—	0.93
d-Pinoresinol (II)	30	—	0.93 *
Group B:			
6-*n*-Propylguaiacol (XX)	31	—	0.93
Barium 3-methoxy-4-hydroxy-5-*n*-propylbenzyl sulphonate (XXI)	*cf.* exptl. part	0.88	—
o-Eugenol (XXII)	32	—	0.87
o-Vanillyl alcohol (XXIII)	*cf.* exptl. part	0.87 **	—
o-Homovanillyl alcohol (XXIV)	»	—	0.88
Barium dehydro-divanillyl di-sulphonate (XXV)	»	0.80 *	—
1-(4-Hydroxy-3-methoxyphenyl)-2-(6-hydroxy-5-methoxy-3-propylphenyl) propane (XXVI) ***	33	—	0.89 *
Group C:			
*trans-Iso*eugenol (XXVII)	34	—	0.69
Coniferyl alcohol (XXVIII)	29	—	0.72
Coniferyl aldehyde (XXIX)	*cf.* exptl. part	0.85	0.88
Ferulic acid (XXX)	—	—	0.92
Group D:			
Vanillin (XXXI)	—	—	0.76 ****
4-Acetoguaiacone (XXXII)	*cf.* exptl. part	—	0.77
4-Propioguaiacone (XXXIII)	»	—	0.74
Vanillic acid (XXXIV)	35	—	0.76

* Based on *one* phenolic equivalent of the substance.
** After a reaction time of 1 minute (*cf.* p. 327).
*** Kindly submitted by Dr. Gunhild Aulin-Erdtman.
**** Not constant after 8 days (*cf.* Fig. 2).

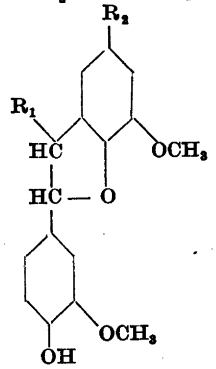
Group A:



IX: R = CH_2OH
X: R = $CHOH-CH_3$
XI: R = $CHOH-CH_2-CH_3$
XII: R = $CH_2OC_2H_5$
XIII: R = $CH(SO_3Ba_{0.5})-CH_2-CH_3$
XIV: R = $CH_2-CH=CH_2$
XV: R = $CH_2-CO-CH_3$

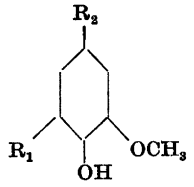


XVI

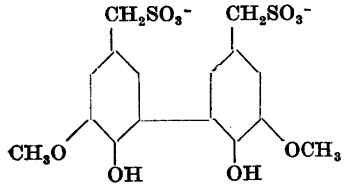


XVII: $R_1 = CH_3$, $R_2 = CH=CH-CH_3$
XVIII: $R_1 = CH_3$, $R_2 = CH_2-CH_2-CH_3$
XIX: $R_1 = CH_2OH$, $R_2 = CH=CH-CH_2OH$

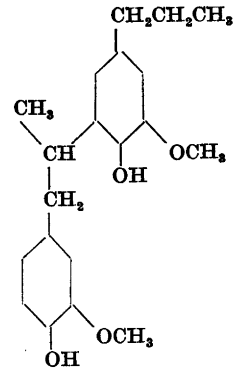
Group B:



XX: $R_1 = CH_2-CH_2-CH_3$, $R_2 = H$
XXI: $R_1 = CH_2-CH_2-CH_3$, $R_2 = CH_2(SO_3Ba_{0.5})$
XXII: $R_1 = CH_2-CH=CH_2$, $R_2 = H$
XXIII: $R_1 = CH_2OH$, $R_2 = H$
XXIV: $R_1 = CH_2-CH_2OH$, $R_2 = H$

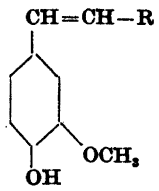


XXV



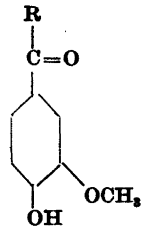
XXVI

Group C:



XXVII: R = CH_3
XXVIII: R = CH_2OH
XXIX: R = CHO
XXX: R = COOH

Group D:



XXXI: R = H
XXXII: R = CH_3
XXXIII: R = CH_2–CH_3
XXXIV: R = OH

alkyl ether (XII), and a sulphonic acid (XIII), it includes the "dimeric" lignin models XVI (a guaiacyl-propane-β-aryl ether), XVII—XIX (coumarans), and *d*-pinoresinol (II).

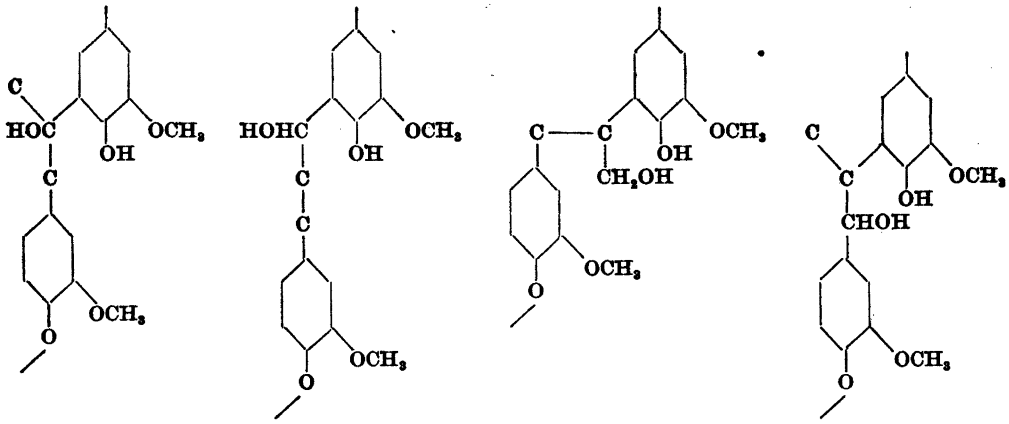
The behaviour of all these substances was very similar to that of guaiacol itself, the rates of methanol formation being practically the same as for guaiacol. The final amounts of methanol ranged from 0.88 to 0.94 moles per guaiacyl nucleus, with a mean value for all substances of 0.91.

It may be noted here that in the periodate oxidation of the carbinols IX, X, and XI the side-chains are partially split off yielding the corresponding aliphatic aldehydes in yields of 50—60 % of the theoretically possible amounts [36].

Group B. In addition to (phenolic or etherified) 4-propylguaiacol residues lignin contains 4,6-substituted guaiacol elements. Predominantly, the phenolic hydroxyl group and the 6-substituent will be joined by ring-closure, as in dehydro-diconiferyl alcohol (XIX), but there are also indications of the occurrence, in lignin, of minor amounts of phenolic structures like those represented by the upper ring of formula XXVI (*cf.* Richtzenhain [37], Aulin-Erdtman [11]). Furthermore, the presence of 6,6'-diguaiacyl structures, represented here by model substance XXV, has been discussed, although recent spectrophotometric evidence indicates [11] that their amount (in lignosulphonic acids) must be very low. Aulin-Erdtman [33] has shown that the two neighbouring phenolic groups in such substances have highly different ionization constants, and it could be supposed that this might influence their reactivity towards periodic acid.

Similarly, it appeared possible that the dehydrogenation of the phenolic hydroxyl group in *o*-vanillyl alcohol (XXIII) might be influenced by intramolecular hydrogen bonding. In lignin, the presence of *o*-guaiacyl carbinol groupings is, however, highly improbable. Tertiary carbinols of type XXXVI seem not to be present in lignin in appreciable amounts [1]. And also, the occurrence of carbinols of type XXXVII, represented by *o*-vanillyl alcohol (XXIII), would be hardly conceivable on the basis of the present views regarding the biosynthesis of lignin.

The main part of the aliphatic hydroxyl groups in lignin are probably primary carbinol groups [1]. Therefore, the presence of phenolic structures



XXXVI XXXVII XXXVIII XXXIX

like XXXVIII appears possible, although, as mentioned above, their frequency in lignin seems to be low [11]. Furthermore, structures like XXXIX cannot be excluded. In order to examine whether *ortho* side-chains with aliphatic hydroxyl groups in the β-position, such as in XXXVIII and XXXIX, may have any effect on the oxidation of the guaiacyl grouping, homo-*o*-vanillyl alcohol (XXIV) was synthesized as a model substance.

The periodate oxidation of the majority of the substances of group B exhibited no peculiarities. 6-*n*-Propylguaiacol (XX) yielded methanol at the same rate and in the same amounts as did guaiacol, and, similarly, the values obtained with substances XXI, XXII, XXIV, and XXVI were on the same level as those presented in group A. The oxidation of the diphenyl derivative XXV was slightly retarded, the final methanol value being reached shortly after a reaction time of five minutes. The extent of side-reactions was somewhat increased which is indicated by the comparatively low yield of methanol, *viz.*, 0.80 mole per guaiacyl nucleus. Possibly, the increased electron density at the *ortho*-linkage between the two phenolic nuclei favours attack of hydroxyl radicals upon the linked ring carbon atoms.

The behaviour of *o*-vanillyl alcohol (XXIII) was unlike that of all the other substances examined. The methanol value given for XXIII in Table 1 refers to a reaction time of only one minute. After a few further minutes of reaction a colourless crystalline solid began to deposit, the amount of which increased during the following hours. As the precipitate appeared, methanol determinations yielded values which were considerably lower (down to 0.4 moles of CH_3OH per mole of XXIII) than the initial value recorded in Table 1. It then was found that an aqueous suspension of the isolated precipitate, when boiled at pH 4.5, yielded a distillate which contained methanol.

The elementary composition of the crystalline solid, m. p. 171—172°, was $C_8H_8O_3$, which agrees to vanillyl alcohol minus two atoms of hydrogen. It contained 20.36 % OCH_3 (calc. OCH_3 20.40). Probably the substance is a dimerization product, $C_{16}H_{16}O_6$. Its structure as well as that of similar products obtained from other *o*-hydroxybenzyl alcohols is being further investigated.

The properties of the solid oxidation product of XXIII explain the observation mentioned above that the methanol values became lower when the amount of deposited

product increased. The deposited product containing the "loosely bound" methoxyl group was removed in the precipitation with lead nitrate, and, therefore, methanol was lost.

The behaviour of XXIII suggested the possibility that, in the periodate treatment, comparatively stable intermediates containing methoxyl groups in some "loosely bound" form might be similarly formed from other guaiacol compounds, although only in the case of XXIII was the intermediate sparingly water-soluble and therefore separated from the reaction mixture. If this were true, serious difficulties might arise in the application of the periodate method to the estimation of phenolic groups in lignin, especially in lignosulphonic acids. Oxidized material, carrying "loosely bound" methoxyl groups, might be totally or partially removed in the lead nitrate precipitation step, and methanol would thus be lost.

This possibility prompted experiments with the aim of examining whether or not the methanol which is obtained, when the filtrate from the lead precipitate is distilled, is generally present in a free state after completed periodate oxidation. Three representatives of group A were investigated in this respect. It was found that, after oxidation of guaiacol and carbinol XI in aqueous solution as well as of XVII in 80 % acetic acid the total methanol content could be distilled off from the reaction mixtures at 3 mm Hg (boiling temperature about 0°). This result, together with the fact that crystals of *cis-cis*-muconic acid deposit from an aqueous solution containing guaiacol and sodium periodate at 4°, constitute sufficient evidence in favour of the view that, generally, methanol is liberated from the guaiacyl nuclei in the oxidation step, and that the different behaviour of *o*-vanillyl alcohol is unusual. This question will be further discussed in a forthcoming publication.

Group C. It is well established that side-chains with a double-bond in conjugation with a phenolic nucleus are not present to any appreciable extent in lignin. This is obvious especially from spectrophotometric data [11], which indicate that mildly prepared lignin products contain less than 0.03—0.04 groups of this type per methoxyl group. On the basis of a specific colour reaction, Lindgren and Mikawa [2] state that small amounts of coniferyl alcohol groupings may be present in native lignin. As yet, however, there is no definite information as to whether such groups may be phenolic or etherified. The coniferyl aldehyde groups demonstrated in lignin [38,39] in an amount of one such group per 40—60 methoxyl groups, have been shown to occur predominantly in the etherified state [11]. Since lignin is devoid of carboxylic groups, the behaviour of the unsaturated substances XXVII—XXIX is of minor importance in the lignin problem.

*Iso*eugenol and coniferyl alcohol (XXVIII) reacted rapidly with periodate. Methanol formation, however, did not reach the normal level, the maximum values being about 0.7 mole of methanol per mole. This is obviously due to oxidative dimerization to dehydro-di*iso*eugenol (XVII) and dehydro-diconiferyl alcohol (XIX), respectively, taking place in competition with the oxidative demethylation and resulting in the blocking of phenolic groups. (*Cf.* the formation of XVII [30] and XIX[29] by ferric chloride oxidation of XXVII and XXVIII, respectively.

Coniferyl aldehyde (XXIX), in which the C=C bond is stabilized by further conjugation with a carbonyl group, probably will not undergo oxidative dimerization and yielded a normal methanol value. The same was true for ferulic acid (XXX).

Group D. As for the substances of group C, the carbonyl compounds collected in group D are of secondary importance as lignin models. It is true that lignin contains a certain number of carbonyl groups. In addition to the

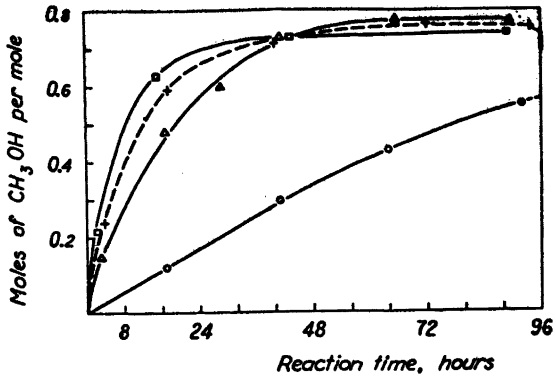


Fig. 2. Formation of methanol on periodate oxidation of O——O *vanillin,* △——△ *4-acetoguaiacone,* □——□ *4-propioguaiacone,* ×– – –× *vanillic acid. (0.5 millimole of substance and 3.5 millimoles of sodium metaperiodate in 50 ml of acetic acid-water (4 : 1), at 4°.)*

coniferyl aldehyde end groups (0.02—0.025 per OCH_3[38]), about 0.1 CO per OCH_3 has been shown to be present in Brauns' "native lignin" [40] and in lignosulphonic acid [41], both from spruce. These carbonyl groups, however, are mainly not in the α-position of the side-chains [42,40]. Consequently, neither vanillin nor the ketones XXXII and XXXIII are appropriate models for the majority of the carbonyl-containing phenolic elements in lignin. Their behaviour may be, however, of some interest, as it may illustrate the structural influences upon the course of the periodate oxidation of guaiacols.

The carbonyl compounds examined are oxidized at a much slower rate than the substances of groups A—C (Fig. 2). In the oxidation of vanillin, methanol was still being formed after one week's reaction, and in the case of the ketones XXXII and XXXIII the methanol values became constant only after about 48 hours. Vanillic acid (XXXIV) behaved in a similar way. It appears likely that this decreased activity towards periodate is due to the +I and +E effects exerted by the substituents and resulting in a diminished electron density at the phenolic hydroxyl group.

The final methanol values obtained in the case of the ketones and vanillic acid are definitely lower than those from the rapidly oxidized substances (except *iso*eugenol and coniferyl alcohol, see above). Since neither α-carbonyl groups nor carboxylic groups seem to be present in lignin in appreciable amounts, this fact is of practically no importance for the application of the method to lignin. In this connection, it may also be pointed out that a carbonyl group in the β-position of the side-chain, as in guaiacylacetone (XV), does not decrease the yield of methanol.

CONCLUSIONS

As an average for the yield of methanol from the various model substances in group A a value of 0.91 moles of methanol per mole is obtained. On the basis of this result and of the preceding discussion regarding the behaviour of

the other model substances, it appears justified to adopt this value as the yield of methanol per phenolic guaiacyl nucleus which can be expected on periodate oxidation of softwood lignin preparations. Consequently, any methanol value obtained (CH_3OH/OCH_3), when multiplied by 1.1, will give the number of phenolic hydroxyl groups per methoxyl in the lignin preparation examined.

In addition to the guaiacyl elements, softwood lignin contains very small amounts of syringyl and *p*-hydroxyphenyl elements [48]. Preliminary experiments have shown that pyrogallol-1,3-dimethylether is partially demethylated by periodate, the yield of methanol being slightly higher than that obtained from guaiacol. Phenolic elements of the *p*-hydroxyphenyl type, which may be present in lignin, are of course not determinable by the periodate demethylation method. The amount of such methoxyl-free elements, however, seems not to exceed 4 % of the total amount of phenyl propane units, and if it is assumed that one fourth of the *p*-hydroxyphenyl elements is phenolic, any figure obtained with the periodate method will be too low by an amount of, at most, 0.01 phenolic groups per methoxyl. In other words, the traces of syringyl and *p*-hydroxyphenyl groups present in softwood lignin will not have any appreciable influence on the estimation of phenolic groups.

EXPERIMENTAL

Periodate oxidation. 1. In aqueous solution. A solution of 0.5 millimole of the substance in 25 ml of water was kept for about 10 minutes at 4° in a dark-room. (Of substances containing two phenolic nuclei, 0.25 millimole was used.) A solution of 3.5 millimoles of sodium metaperiodate, $NaIO_4$, in 25 ml of water, cooled to 4°, was added. The reaction was stopped by the addition of 25 ml of 20 % lead nitrate solution. The precipitate (lead iodate and periodate) was filtered off and washed thoroughly with water.

From the filtrate (about 200 ml) about 100 ml were distilled off in a slow stream of nitrogen and the distillate was collected in an ice-cooled receiver containing about 50 ml of water. In order to remove traces of iodine, the distillate was passed through a column (13 × 200 mm) of an anion exchange resin (Dowex 2), which had been saturated with bisulphite ions. The column was washed with water, and the filtrate made up to a final volume of 300 ml.

Formaldehyde which is formed in the oxidation of vanillyl alcohol (IX) and which would interfere with the subsequent methanol determination, is completely retained by the bisulphite-saturated resin [44] in the same operation.

2. In 80 % acetic acid. Substances which were sparingly soluble in water, were oxidized in 80 % acetic acid. Glacial acetic acid *pro analysi*, 99—100 % (E. Merck, Darmstadt), which was shown to be free of methanol, was used.

The solution of the substance (0.5 millimole) in 25 ml of glacial acetic acid was mixed with 25 ml of a solution of sodium metaperiodate, prepared by dissolving 0.14 mole of periodate in 400 ml of water and adding acetic acid to a volume of 1 litre. The oxidation was carried out at 4° in a dark-room. At the end of the reaction time 25 ml of a 20 % aqueous lead nitrate solution was added, the mixture filtered and the precipitate washed with about 50 ml of acetic acid and subsequently with about 75 ml of water.

The combined filtrates were distilled as described above. As it was found that acetic acid, when oxidized with permanganate under the conditions used in the following oxidation step, gave rise to formaldehyde, acetic acid contained in the distillate had to be removed. For this purpose the distillate was made alkaline by the addition of an excess of 50 % sodium hydroxide solution, and redistilled. The final distillate was made up to a volume of 300 ml.

If formaldehyde was formed during the periodate oxidation, the final distillate was passed through a bisulphite-saturated anion exchange resin (see above), and the filtrate made up to a volume of 300 ml.

Determination of methanol. The procedure of Ahlén and Samuelson [44] was followed with slight modifications. To a mixture of 25 ml of the distillate, obtained according to procedure 1 or 2 above, and 5 ml of an aqueous solution of phosphoric acid (165 ml H_3PO_4, $d = 1.75$, per litre), 5 ml of potassium permanganate (40 g $KMnO_4$ per litre) were added at 25°. After exactly 10 minutes, 10 ml of a mixture of oxalic and sulphuric acid (40 g $H_2C_2O_4$, $2H_2O$ and 40 ml of conc. H_2SO_4 per litre) were added. The solution became colourless after about 10 minutes. The carbon dioxide evolved was removed by shaking, and 5 ml of the solution were mixed with 5 ml of a freshly prepared 2 % aqueous solution of chromotropic acid and 25 ml of conc. sulphuric acid. This mixture was heated in a loosely stoppered Erlenmeyer flask for 45 minutes in a boiling water bath. The extinction of the solution was measured at 570 mμ against a blank solution prepared in a similar way from 25 ml of distilled water.

A standard curve was obtained with aqueous solutions of methanol of known concentration, which were treated as described above. At least up to a concentration of 30 mg of methanol per 300 ml the amount of formaldehyde formed is a linear function of the methanol content.

The accuracy of the method may be illustrated by the following example (periodate oxidation of guaiacol, *cf.* Fig. 1).

Time of periodate oxidation, hrs	1/60	1/4	1/2	1	6	14	38	72
Moles of CH_3OH per mole of guaiacol	0.89	0.92	0.92	0.91	0.92	0.93	0.92	0.91

cis-cis-Muconic acid from guaiacol. From the red-brown aqueous solution obtained when guaiacol was oxidized with periodate under the conditions given above, a carboxylic acid could be isolated by extraction with ethyl acetate. The crystalline product, after recrystallization from water, had a wide melting point range and probably consisted of a mixture of the geometric isomers of muconic acid.

If the concentration of guaiacol was increased to 0.8 millimole per 25 ml of water and the amount of periodate kept unchanged, a small amount of nearly colourless prismatic needles deposited from the reaction mixture after some hours at 4°. The crystals were collected and washed with little water; m. p. 187°, no depression with *cis-cis*-muconic acid, m. p. 187—188°, prepared from phenol or pyrocatechol and peracetic acid [21].

α-(4-Hydroxy-3-methoxyphenyl)-ethanol (=Apocynol) (X). Sodium borohydride (0.45 g) was added to a solution of 4-acetoguaiacone (4 g) and sodium hydroxide (1.2 g) in water (60 ml). After three days at 60° a sample of the mixture no longer gave any precipitate with 2,4-dinitrophenylhydrazine. The solution was neutralized at room temperature with carbon dioxide, and extracted with ether. Evaporation of the dried ether solution yielded 3.7 g of a crystalline product, which was recrystallized by dissolving in ethylacetate (without heating!) and precipitation with hexane. M. p. 101—102° C (101° according to Finnemore [45]).

Vanillyl ethyl ether (XII). A 4 % solution of dry hydrogen chloride in abs. ethanol (521 g) was added dropwise during 3 hours to a stirred, ice-cooled solution of vanillyl alcohol (30 g) in abs. ethanol (500 g). After 16 hours at room temperature, somewhat less than the equivalent amount of sodium hydroxide (15 g NaOH in 50 ml H_2O) was added and the solution finally neutralized with a saturated sodium bicarbonate solution. Ethanol was distilled off and the residue extracted with chloroform. The chloroform solution was dried over Na_2SO_4, and the solvent removed by distillation. The residual oil was distilled, b. p. 124—125° at 4 mm; $n_D^{20} = 1.5297$. Yield 85 %. (Found: C 65.6; H 7.59; alkoxyl calc. as OCH_3 33.9. Calc. for $C_{10}H_{14}O_3$: C 65.9; H 7.74; alkoxyl calc. as OCH_3 34.1.)

Barium 1-(4-hydroxy-3-methoxyphenyl)-n-propane-1-sulphonate (XIII) *. Guaiacyl ethyl carbinol (XI) (1 g) was sulphonated with an aqueous solution (50 ml) containing 14.3 g NaOH and 50 g SO_2 per litre. The mixture (pH 1.4) was heated to 135° during 3 hours and kept at this temperature for a further 3 hours. Excess of SO_2 was removed *in vacuo*, the solution neutralized with barium carbonate and filtered. The filtrate was

* This preparation was carried out by Mr. E. Eriksoo.

passed through a cation exchange resin and sulphur dioxide removed *in vacuo*. The solution was reneutralized with calcium carbonate and the precipitate ($CaSO_3$) filtered off. Residual sulphite was precipitated by the addition of saturated calcium hydroxide solution to pH 8—9. The filtered solution was concentrated *in vacuo* in a carbon dioxide atmosphere to about 40 ml, the concentrate filtered and again passed through a cation exchange resin. Finally, the solution was neutralized with barium carbonate, filtered and concentrated *in vacuo* to a small volume. The barium salt (XIII) deposited and was recrystallized by dissolution in *iso*propanol-water (85 : 15) and addition of ethyl ether. Colourless needles. (Found: OCH_3 9.55; S 10.47; Ba 21.4. Calc. for $C_{10}H_{13}O_5SBa_{0.5}$: OCH_3 9.85; S 10.18; Ba 21.8.)

5-n-Propylvanillyl alcohol. 5-Allylvanillin [32] (5 g), dissolved in 96 % ethanol (25 ml) was shaken with platinum oxide catalyst (0.05 g) in a hydrogen atmosphere at a pressure of 4 kg/cm^2. One mole of hydrogen per mole of the substance was rapidly consumed. Since 2,4-dinitrophenylhydrazine produced a precipitate with a sample of the solution, only the allyl group had been hydrogenated. A further mole of hydrogen was consumed after the addition of 0.5 ml 1 *M* $FeCl_3$, and the dinitrophenylhydrazine test became negative. The catalyst was filtered off and the solvent removed *in vacuo*. The residual oil was dissolved in ether, the solution dried over Na_2SO_4 and the solvent evaporated. The product crystallized from benzene-hexane in needles, m. p. 45—47°. Yield 4.5 g (88 %). (Found: OCH_3 15.97; Calc. for $C_{11}H_{16}O_3$: OCH_3 15.81.)

The free phenol alcohol was unstable at room temperature; it turned slightly yellow and became liquid after a few days. After a few weeks the product had crystallized again. Recrystallization from ethanol-water yielded the diphenylmethane described below.

Bis-(4-hydroxy-3-methoxy-5-n-propylphenyl-)methane. Attempts to prepare 5-*n*-propylvanillyl alcohol from 6-*n*-propylguaiacol (XX) by the Lederer-Manasse reaction yielded the corresponding diphenylmethane. A mixture of 6-*n*-propylguaiacol (5 g), sodium hydroxide (6 g NaOH in 30 ml H_2O) and formalin (8 ml) was kept at 40—45° for one week. Neutralization with carbon dioxide yielded a white precipitate, which after washing with petroleum ether was recrystallized from ethanol-water. Needles, m.p. 124—125°; yield 1.4 g. (Found: C 73.3; H 8.26; OCH_3 18.22. Calc. for $C_{21}H_{28}O_4$: C 73.2; H 8.19; OCH_3 18.02.)

Barium 3-methoxy-4-hydroxy-5-n-propylbenzyl sulphonate (XXI). Freshly prepared 5-*n*-propylvanillyl alcohol was heated at 135° for three hours with an aqueous solution of sodium sulphite and sodium bisulphite of pH 6.6 and a total SO_2 content of 5 %. The sulphonic acid formed was isolated as described above for XIII. Needles from *iso*propanol-water (85 : 15)-ethyl ether. (Found: OCH_3 9.42; Ba 20.8. Calc. for $C_{11}H_{15}O_5SBa_{0.5}$: OCH_3 9.46; Ba 20.9.)

o-Vanillyl alcohol (XXIII). *o*-Vanillin was reduced catalytically as described by Carothers and Adams [33] for other hydroxybenzaldehydes. The oily product crystallized in needles from benzene-hexane. Yield 91 %, m. p. 60—61° (60—62° according to Eliel [46]).

o-Homovanillyl alcohol (XXIV). *o*-Eugenol (5.0 g) was treated in ethyl acetate solution with a stream of oxygen containing 0.8—1.0 % of ozone, as described by Hahn and Schales [47] for the ozonisation of safrol. Hydrogenation with Pd-$CaCO_3$ catalyst and bisulphite extraction yielded 4.0 g of a crystalline bisulphite addition product. This was dissolved in water and the solution extracted with ether to remove impurities. Sodium hydroxide (4 g) and sodium borohydride (1 g) were added to the aqueous phase. The mixture was kept over night at 50°, neutralized with carbon dioxide and extracted with ethyl ether. The ether solution was dried over Na_2SO_4 and the ether evaporated, yielding a colourless, viscous oil. Yield: 1.6 g. (Found: OCH_3 18.37; Calc. for $C_9H_{12}O_3$: OCH_3 18.45.)

Dehydrodi-vanillyl alcohol. Dehydrodi-vanillin (8.2 g), dissolved in sodium hydroxide (6.5 g NaOH in 60 ml H_2O) was reduced with sodium borohydride (1.0 g) at room temperature. After 16 hours, long needles of the sodium phenolate had deposited. These were collected, dissolved in water (200 ml), and the free phenol was precipitated with acetic acid. Recrystallization from ethyl acetate yielded 6.42 g (76 %) of a crude product, which was purified by recrystallization from acetone-ethanol (1:1). Prisms, m. p. 187—190° (rapid heating). (Found: C 62.7; H 6.11; OCH_3 20.6. Calc. for $C_{16}H_{18}O_6$: C 62.7; H 5.93; OCH_3 20.3.)

Barium dehydrodi-vanillyl disulphonate (XXV). The sulphonation of dehydrodi-vanillyl alcohol was carried out as described for the preparation of the sulphonate XIII.

The barium salt deposited from the final aqueous concentrate and was completely precipitated by the addition of ethanol and ether. Reprecipitated from aqueous *iso*propanol-ethyl ether. (Found: OCH_3 10.74; Ba 23.8. Calc. for $C_{16}H_{16}O_{10}S_2Ba$: OCH_3 10.89; Ba 24.1.)

Coniferyl aldehyde (XXIX) was prepared according to Pauly and Feuerstein [49] with a few modifications *. The condensation of methoxymethyl vanillin with acetaldehyde was carried out in a borate buffer solution at pH 11.5. Coniferyl aldehyde was obtained from its methoxymethyl ether by heating the latter in a solution of 0.1 % conc. H_2SO_4 in 50 % acetic acid for 1 hour at 80° in a nitrogen atmosphere.

4-Acetoguaiacone (XXXII) **. The substance was prepared from guaiacol, acetic acid, and boron fluoride, following the procedure used by Kästner [48] for the synthesis of *p*-hydroxyacetophenone. Its purification was carried out according to Reichstein [50]. Yield 50 %.

4-Propioguaiacone (XXXIII). From guaiacol, propionic acid, and boron fluoride. Yield 60 %, m. p. 58° (*cf.* Kratzl [51]).

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** Substances XXXII and XXXIII were prepared by Mr. S. Delin.

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Short Communications

The Distribution of Sulphur in Cytochrome c

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In connection with other investigations [1] we have found it necessary to reinvestigate the distribution of sulphur in cytochrome c. In a recent review [2] it was concluded that the cytochrome c molecule contains six sulphur atoms, two of which belong to methionine [3], two to the cysteine residues in the thioether bridges [4,5], and two to cystine. These conclusions were based on data obtained on cytochrome c preparations purified according to Theorell and Åkeson [6] with the use of electrophoresis at different pH levels. We have now redetermined the content of sulphur and methionine in cytochrome c prepared on ion exchangers [7,8] and tested the cytochrome c for the presence of sulfhydryl and disulphide groups.

Experimental and results. Cytochrome c preparation. Cytochrome c was prepared from beef hearts on ion exchangers according to the principles given by Paléus and Neilands [7] and Margoliash [8]. The preparation contained 0.429 % iron.

Sulphur analysis. Three different methods were used, namely those of Josephson [9], Kirsten [10] and Zimmermann [11]. The figures show that cytochrome c contains 4 atoms of sulphur per molecule. The values obtained with the methods of Kirsten and Zimmermann agree very well, while the method of Josephson seems to give too low values (Table 1).

Determination of methionine. Methionine was determined by a modification of the Baernstein method [12]. We have only determined the methyl iodide formed and not the homocysteine thiolactone, which is the other product of the digestion. The determination was carried out in a Pregl micromethoxyl apparatus according to Vieböck and Brecher [13]. The yield of methyl iodide for a pure sample of methionine was found to be 96 % and the corresponding factor was thus 1.04. When 40.6 mg of cytochrome c with the iron content of 0.429 % was digested with hydriodic acid for 12 hours 0.922 mg CH_3I was obtained, corresponding to 2.16 methionine residues per molecule cytochrome c. Cytochrome c thus contains two methionine residues in the molecule.

Sulfhydryl- and disulphide group determination. The sulfhydryl group determination in the intact cytochrome was carried out with the mercurimetric amperometric titration method as published by Kolthoff *et al.*[14]. No mercury consumption could be detected in up to 28.5 mg of preparation used (2.4×10^{-6} moles cytochrome c). As this method determines as little as 5×10^{-8} moles of cysteine, cysteine must be absent from cytochrome c. The native cytochrome and preparations hydrolyzed in 20 % HCl for up to 20 hrs were titrated in the presence of sulphite with mercury ions according to Stricks *et al.*[15]. Up to 35 mg hydrolyzed cytochrome (2.9×10^{-6} moles) was titrated but in none of the samples could mercury consumption be demonstrated. As the sensitivity in this case is about 2×10^{-7} moles cystine, less than 1/10 mole of cystine is present in 1 mole of cytochrome c.

In addition, the nitroprusside test for sulfhydryl groups was negative on the preparation used in this work. This confirms the results obtained by Theorell and Åkeson [16]. The nitroprusside test for disulphide groups [17] was also negative.

Åkeson [3] found two methionine residues and two cysteines involved in the thioether linkages to the heme prosthetic group and could thus account for the presence of four of the six S atoms/molecule. The methionine analysis in the present work only verifies the data of Åkeson. The sulphur analyses, however, have revealed only four

Table 1. Results of the sulphur analyses.

Preparation	% Sulphur	Methods	Atoms sulphur per mole cyt. c (calculated from the iron content)
0.429 % iron	0.87, 0.87	Josephson	3.52
0.429 » »	1.00, 0.95	Kirsten	3.97
0.429 » »	0.94	Zimmermann	3.81
0.409 » » *	0.98	»	4.17

* This preparation was kindly provided by Drs H. Tuppy and G. Bodo, II. chem. Laboratorium der Universität, Wien.

sulphur atoms/molecule, and we have therefore been able to account for all the sulphur occurring in this preparation of cytochrome c.

The discrepancy of the present work with the results of Carruthers [18] is difficult to explain. Using electrophoretically purified cytochrome c with an iron content of 0.411 % he found a catalytic double wave in an ammoniacal solution of hexamine cobalt ions, which he attributed to cysteine or cystine in the cytochrome. There is a possibility that the cobalt ions present in the experiments of Carruthers might split the thioether bond of cytochrome c in the same way as silver ions [19], the double catalytic wave thus being caused by the two heme-linked cysteine residues.

Paul [20] dissociated a specially purified preparation of cytochrome c (iron content = 0.424 %) with silver and found that the protein moiety combined with 3.5—3.6 atoms of silver, probably due to two thiol groups (derived from the thioether bridges) and one disulphide group.

There is still the difference of two sulphur atoms between the preparation of Theorell and Åkeson [16] and the preparation of the present work to be explained. Perhaps that difference might be due to the preparation method used in each case. Further investigations of this difference would be of great interest.

Acknowledgements. The author wants to express his sincere gratitude to Professor H. Theorell and Dr. B. Sörbo for kind advice and criticism in connection with the work.

It is a pleasure to thank Dr. G. Kainz and Mr. W. Silhan at the II. Chemical Laboratory of the University of Vienna for careful analyses.

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Organic Hydroxylamine Compounds Formed from Nitrite in *Torulopsis utilis*

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For some twenty years the formation of hydroxylamine both in the reduction of nitrate and in the fixation of molecular nitrogen has often been dealt with [1]. Bound hydroxylamine has been found to be formed in connection with both processes ("bound hydroxylamine" = a substance released on acid hydrolysis, which on oxidation with iodine gives nitrite [2]). On the whole, the nature of the "bound hydroxylamine" has remained unknown. Only Virtanen and Laine [3] were once able to isolate an oxime compound from the "sterile" sand-substrate of pea inoculated with effective *Rhizobium*, which compound on reduction gave aspartic acid and accordingly was, or contained, the oxime of oxalacetic acid. Except oxime nitrogen "bound hydroxylamine" could also be hydroxamic acid, especially as the formation of hydroxamic acid from hydroxylamine and organic acids or their amides has been brought about with preparations made from plant- and animal organisms.

Chromatographic methods have rendered it possible to study the "bound hydroxylamine" fraction more closely. Work on this line is going on in our laboratory. The first results of these investigations are presented in this communication.

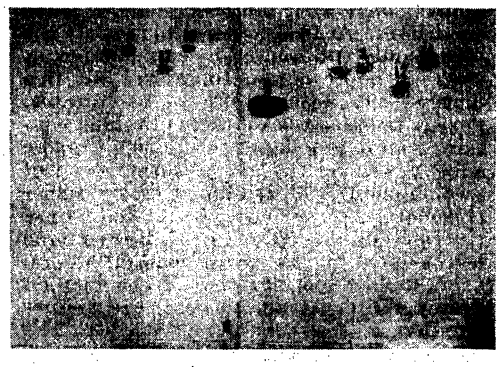
Virtanen and Csáky [4] observed that both low- and normal-nitrogen *Torulopsis utilis*, when suspended in a nitrate containing solution, which was vigorously aerated, forms "bound hydroxylamine" very rapidly. The amount of it decreased, however, as soon as after 15—20 min., probably indicating that the enzyme needed for its reduction had time to become effective enough through adaptation. We therefore used *Torulopsis*-yeast in our experiments. As the strain used now formed only a very small amount of "bound hydroxylamine" from nitrate as well as from nitrite in aerated solutions we performed our experiments in a low oxygen solution containing nitrite, the amount of "bound hydroxylamine" increasing noticeably under these conditions. At first we tried to identify the compounds containing hydroxylamine as such by paper chromatography, but this method did not turn out to be suitable, mostly because the extracts obtained contained large amounts of unknown organic substances. As the identification even of small amounts of amino acids by paper chromatography is easy we started to use the method of reducing hydroxylamine compounds to the corresponding amino acids and to identify these. The method presupposes that amino acids are beforehand removed as carefully as possibly. The method we used was briefly the following:

115 g of fresh low-nitrogen *Torulopsis utilis* (N-content 6.0 % of dry materia) was suspended in 1 150 ml of a mineral nutrient solution without combined nitrogen in a Kluyver flask placed in a waterbath at 30° C. Air was flushed through the suspension for 10 min., followed by nitrogen gas for 10 min. more before the addition of sodium nitrite in a concentration of 75 μg N/ml. This leads to the accumulation of about 3 μg NH_2OH-N/g fresh yeast. After 35 min. the suspension was centrifuged, the cells were washed with icecold distilled water, re-centrifuged and suspended in 96 % alcohol (3 ml/g yeast) freed from aldehydes. The whole operation after the addition of nitrite took 1 h. The suspension was kept in an icebox overnight and then centrifuged.

The clear supernatant was evaporated *in vacuo*, lipid material extracted by *iso*amylalcohol, and amino acids removed by cation exchange resin (Dowex 50) in the cold (0—3°C). The solution then contained 240 μg bound hydroxylamine-N, but no nitrite or free NH_2OH. Only traces of some amino acids could be found in the solution (*cf.* below). Half of the solution was subjected to reduction by 1.5 % sodium amalgam in the cold, the other half regarded as a control. Reduction time was 6 h whereby about 70 % of bound hydroxylamine was reduced.

Amino acids were absorbed on Dowex 50, displaced by ammonia and chromatographed on Whatman No. 4 paper using the solvent system butanol-acetic acid and phenol-NH_3. As a further control 73 g of *Torulopsis*-yeast were treated in the same way, but without addition of nitrite (Figs. 2A and 2B).

Figs. 1 and 2 show a two-dimensional paper chromatogram from the experiment in which nitrite had been added to the suspension of *Torulopsis*-yeast. Fig. 1 A shows the amounts of amino acids *before*

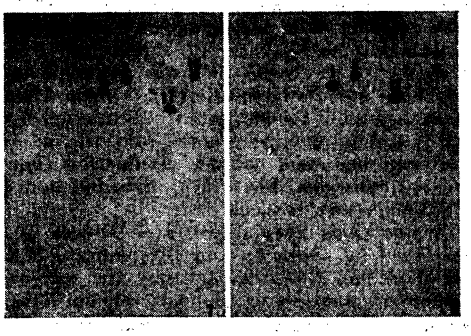


A B

Fig. 1 A. Two-dimensional paper chromatogram from the experiment with Torulopsis utilis suspension in a nitrite containing solution after removing of amino acids and before reduction. Only traces of 1 = gly, 16 = asp, 17 = glu, distinctly more of 8 = ser.

B. The same as A but after reduction with sodium amalgam. 2 = ala.

reduction, Fig. 1 B again *after* it. It appears from the chromatogram Fig. 1 A that we had succeeded fairly well in removing amino acids from the solution before reduction. Alanine was not found at all before reduction, of glutamic acid, aspartic acid, and glycine only traces were found. Of serine there was a somewhat larger amount. The reduction led to a profuse



A B

Fig. 2 A. Two-dimensional paper chromatogram from the experiment with Torulopsis utilis suspension in a solution without nitrite after removing of amino acids and before reduction. Only traces of 1, 8, 16, 17.

B. The same as A but after reduction.

formation of alanine, and increased the amount of glutamic acid more than tenfold. Similarly the amount of aspartic acid and glycine increased manifold. The amount of serine increased only slightly. It could thus be established that when *Torulopsis* reduces nitrite the oximes of pyruvic acid, ketoglutaric acid, oxalacetic acid, and glyoxalic acid are formed. The formation of the oxime of hydroxypyruvic acid was uncertain. As the keto acids corresponding to these oximes are of common appearance in cells, and hydroxylamine reacts with them with different velocities (Virtanen and Alfthan [5]), it is probable that in the cells hydroxylamine forms oximes with keto acids in amounts corresponding to their respective reaction velocities and concentrations.

The results related show that oximes of α-keto acids are formed in *Torulopsis*-yeasts on the reduction of nitrite. The identification of these have given conclusive evidence concerning the nature of the mysterious "bound hydroxylamine". The results give support to the idea (Virtanen *et al.* [3,5,6]) that when nitrate is the source of nitrogen the formation of amino acids in green plants as well as in micro-organisms may occur along two different pathways: *via* ammonia and *via* hydroxylamine. The reduction of oximino acids in organisms is still fairly unknown.

$$NO_3 \rightarrow NO_2 \rightarrow \rightarrow NH_2OH \rightarrow NH_3$$

$$\begin{array}{lll} & + & + \\ & \text{α-keto acid} & \text{α-keto acid} \\ & \downarrow & \downarrow \\ \text{oxime of α-keto acid} & \rightarrow & \text{α-amino acid} \end{array}$$

Even if the formation of the oximes in cells according to the foregoing obviously is a non-enzymatic reaction, the amino acid synthesis may, along this pathway, lead to a normal amino acid composition as amino acids through the action of transaminases can change into one another. How great a part of amino acids are formed *via* oximes is unknown.

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A Diffusion-equilibration Method as a Stage in the Determination of the Deuterium Oxide Content in 1—2 Microlitres of Fluid

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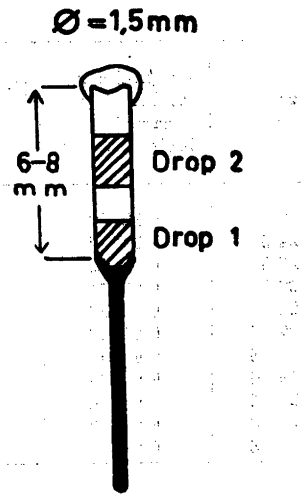
Determination of the deuterium oxide content in fluids is generally performed utilizing the difference in density between pure water and deuterium oxide. Convenient methods have been described which allow an estimation of the deuterium oxide content in very small volumes with sufficient accuracy for most biological purposes. Thus the gradient tube method developed by Linderstrøm-Lang [1] (see also Linderstrøm-Lang, Jacobsen and Johansen [2]) requires only about 0.5 microlitre for an accurate determination. However, all methods based upon density determinations require that the fluid, the deuterium oxide content of which will be determined, contains only H_2O, HDO and D_2O, respectively. A distillation procedure is thus a necessary step before the actual measurements can be made. If the volume of the fluid to be analyzed is limited to a few microlitres, common distillation procedures become inconvenient.

The present work is a description of a simple method for "purification" of very small samples (1—2 μl) prior to the density determination in the gradient tube.

Theory. The principle of the method is shown in Fig. 1. Sample 1, a drop of volume V_1 containing an unknown concentration C_1° of deuterium oxide, is allowed to attain diffusion-equilibrium in a



small closed space with sample 2, a drop of volume V_2 containing distilled water. (It is assumed, for the sake of simplicity, that the distilled water used contains no deuterium oxide. This assumption introduces a negligible error.) The air space between the drops acts as a semipermeable membrane allowing only volatile substances to pass through. After equilibrium has been attained, any volatile substance has the same activity in both drops. It may be assumed that the activity coefficients of HDO (and D_2O) in water do not significantly differ from the activity coefficients of HDO (and D_2O) in water solutions of the molar and ionic strength present in biological fluids. Therefore

$$C_1^{eq} = C_2^{eq} \qquad (1)$$

where the superscripts denote that the concentrations are those at equilibrium.

Furthermore

$$C_1^\circ \, V_1 = (V_1 + V_2) \, C_2^{eq} \qquad (2)$$

and

$$C_2^{eq} = C_1^\circ \frac{V_1}{V_1 + V_2} \qquad (3)$$

If the only volatile substance present is deuterium oxide, a density determination of sample 2 at equilibrium will give the original concentration of deuterium oxide in sample 1. Equation (2) contains of course the assumption that the amount of deuterium oxide present in the air space

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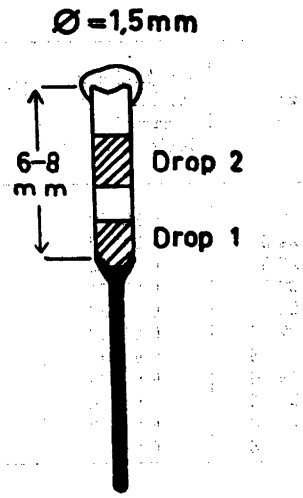

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If the only volatile substance present is deuterium oxide, a density determination of sample 2 at equilibrium will give the original concentration of deuterium oxide in sample 1. Equation (2) contains of course the assumption that the amount of deuterium oxide present in the air space

Table 1.

Expt. No.	Original conc. of HDO in sample 1, Mole fraction	Equilibration time, Minutes	Conc. of HDO in sample 1 after equilibration, Mole fraction	Conc. of DHO in sample 1 (calc.), Mole fraction	Recovery %
17.9	0.12298	1 110	0.061494	0.12299	100.0
24.9	0.12298	140	0.061342	0.12268	99.8
25.9	0.12298	160	0.061368	0.12274	99.8
28.9	0.07074	245	0.036004	0.07201	101.8
29.9	0.02337	300	0.011902	0.02380	101.9
2.10	0.02337	125	0.011664	0.02333	99.8
5.10	0.02337	285	0.011802	0.02360	101.0
20.10	0.01774	300	0.008918	0.01784	100.6
21.10	0.01774	180	0.008960	0.01792	101.0
22.10	0.00912	135	0.004520	0.00904	99.1
23.10	0.00912	160	0.004635	0.00927	101.6

is negligible in comparison with that present in V_1 and V_2.

The basic principle of this method has long been known (*cf.* Linderstrøm-Lang and Holter [3] and Conway [4]).

Method. Small glass tubes of the form and dimensions given in Fig. 1 are made from ordinary glass. They are thoroughly cleaned and treated on the inside with Desicote (Beckman) to make the surface non-wettable. After Desicoting they are again rinsed several times with distilled water. The rinsing after the Desicoting procedure is important as it removes traces of water-soluble matter in the Desicote film. The sample to be analyzed is then transferred into the bottom of the tube, preferably by means of a constriction pipette. Sample 2 is then immediately placed in the tube about 1 mm above the bottom drop (see Fig. 1). These operations are conveniently performed using the clamps and stands for pipettes and tubes described by Holter [5] for Cartesian diver manometry. Immediately after sample 2 has been delivered, the open end of the tube is dipped into melted paraffin which upon rapid cooling forms an effective seal. The tube is then allowed to stand in room temperature for equilibration. The time necessary for complete equilibration depends of course on the dimensions of the system. The dimensions used in the present investigation are seen in Fig. 1 and complete equilibrium is attained after about 100 minutes.

Results. The results from measurements on different standard solutions containing varying amounts of deuterium oxide is seen in Table 1. The concentrations are given as mole fractions HDO as if no D_2O molecules are present, an assumption which is nearly true in the dilutions used. The volume of the samples was 1.6 µl throughout, the same pipette used for both samples. The time for equilibration is also shown, it was always more than 125 minutes. In one instance it was as long as 1 110 minutes without affecting the recovery percentage. The mean recovery percentage from the 11 experiments shown in the table was 100.6 with a range between 99.1 and 101.9.

Discussion. As is seen from Table 1, recovery is obtained with an accuracy sufficient for most biological purposes. It seems therefore that the method described might be useful in those cases where the amount of fluid available for analyses is of the order of a few microlitres. Of course, any volatile substance present in addition to deuterium oxide is liable to affect the density of the recipient drop, and in certain cases this effect might be larger than otherwise tolerated.

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5. Holter, H. *Compt. rend. trav. Lab. Carlsberg, Sér. chim.* **24** (1943) 399.

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Guaiacylglycerol and its β-Guaiacyl Ether

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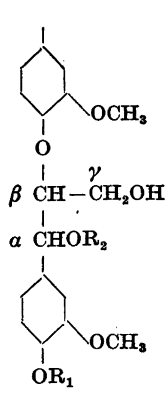
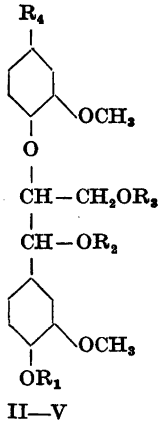
According to the views expressed by Erdtman [1,2] regarding the course of the dehydrogenative dimerization and polymerization of *p*-propenylphenols, guaiacylglycerol-β-aryl ether structures (I), in addition to other structures, can be expected to arise when coniferyl alcohol is subjected to dehydrogenation. The same author discussed the occurrence of such structures in lignin. On the basis of experimental results concerning the behaviour of lignin in sulphonation with sulphite solutions, alkylation with alcoholic hydrochloric acid, and similar reactions, Adler and Lindgren [3,4] and Adler and Yllner [5] also suggested the presence of structure I in lignin.



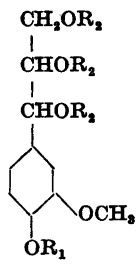
I



II—V

I. R_1 = H or the β-C-atom of a propane side-chain; R_2 = H or a C-atom of a propane side-chain
II. $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$
III. $R_1 = R_2 = R_3 = R_4 = H$
IV. $R_1 = R_2 = R_3 = COCH_3$; $R_4 = H$
V. $R_1 = R_2 = R_3 = H$; $R_4 = CH{=}CH{-}CH_2OH$

The β-guaiacyl ether of α-(3,4-dimethoxyphenyl)-glycerol (II) was synthesized [3] and found to be a promising lignin model. It was sulphonated [3,4] and alkylated [3-6] in the expected manner, and also yielded formaldehyde on heating with strong sulphuric acid [3,4,7]. As in the case of α-(3,4-dimethoxyphenyl)-glycerol (VI) [5,7], the β-guaiacyl ether II yielded Hibbert's "ethanolysis" products on prolonged heating with ethanolic hydrochloric acid. This reaction cannot be given by other dimeric systems like those present in dehydro-diconiferyl alcohol or in pinoresinol which also are assumed to occur in lignin (*cf.* Ref.[5]).



VI. $R_1 = CH_3$; $R_2 = H$
VII. $R_1 = R_2 = H$
VIII. $R_1 = R_2 = COCH_3$

Only one phenolic guaiacylglycerol compound, *viz.*, "guaiacylglycerol" itself, *i. e.*, α-(3-methoxy-4-hydroxyphenyl)-glycerol (VII), has been available as yet. It had been obtained [8] as a syrupy mixture of the two possible pairs of optical antipodes, and yielded two crystalline tetraacetates (VIII), m.p. 84—85° and 113—114°, respectively. A sample of the syrupy product, which contained some water and had been kept in the refrigerator for several months, has now partly crystallized. Inoculation of other samples with the crystalline material induced rapid crystallization. Recrystallization from ethyl acetate, which was saturated with water, yielded needles, m. p. 82—84°. This compound contained one mole of water, which was removed at 60° (0.1 mm Hg). (Found: H_2O 7.71. Calc. for $C_{10}H_{14}O_5,H_2O$: H_2O 7.76.) The anhydrous product was a colourless glass. (Found: C 56.0; H 6.62; OCH_3 14.8. Calc.

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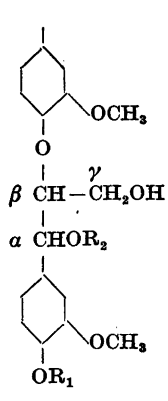
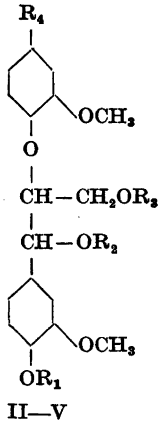
According to the views expressed by Erdtman [1,2] regarding the course of the dehydrogenative dimerization and polymerization of *p*-propenylphenols, guaiacylglycerol-β-aryl ether structures (I), in addition to other structures, can be expected to arise when coniferyl alcohol is subjected to dehydrogenation. The same author discussed the occurrence of such structures in lignin. On the basis of experimental results concerning the behaviour of lignin in sulphonation with sulphite solutions, alkylation with alcoholic hydrochloric acid, and similar reactions, Adler and Lindgren [3,4] and Adler and Yllner [5] also suggested the presence of structure I in lignin.



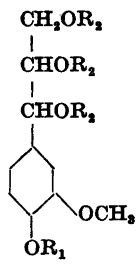
I



II—V

I. R_1 = H or the β-C-atom of a propane side-chain; R_2 = H or a C-atom of a propane side-chain
II. $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$
III. $R_1 = R_2 = R_3 = R_4 = H$
IV. $R_1 = R_2 = R_3 = COCH_3$; $R_4 = H$
V. $R_1 = R_2 = R_3 = H$; $R_4 = CH{=}CH{-}CH_2OH$

The β-guaiacyl ether of α-(3,4-dimethoxyphenyl)-glycerol (II) was synthesized [3] and found to be a promising lignin model. It was sulphonated [3,4] and alkylated [3-6] in the expected manner, and also yielded formaldehyde on heating with strong sulphuric acid [3,4,7]. As in the case of α-(3,4-dimethoxyphenyl)-glycerol (VI) [5,7], the β-guaiacyl ether II yielded Hibbert's "ethanolysis" products on prolonged heating with ethanolic hydrochloric acid. This reaction cannot be given by other dimeric systems like those present in dehydro-diconiferyl alcohol or in pinoresinol which also are assumed to occur in lignin (*cf.* Ref.[5]).



VI. $R_1 = CH_3$; $R_2 = H$
VII. $R_1 = R_2 = H$
VIII. $R_1 = R_2 = COCH_3$

Only one phenolic guaiacylglycerol compound, *viz.*, "guaiacylglycerol" itself, *i. e.*, α-(3-methoxy-4-hydroxyphenyl)-glycerol (VII), has been available as yet. It had been obtained [8] as a syrupy mixture of the two possible pairs of optical antipodes, and yielded two crystalline tetraacetates (VIII), m.p. 84—85° and 113—114°, respectively. A sample of the syrupy product, which contained some water and had been kept in the refrigerator for several months, has now partly crystallized. Inoculation of other samples with the crystalline material induced rapid crystallization. Recrystallization from ethyl acetate, which was saturated with water, yielded needles, m. p. 82—84°. This compound contained one mole of water, which was removed at 60° (0.1 mm Hg). (Found: H_2O 7.71. Calc. for $C_{10}H_{14}O_5,H_2O$: H_2O 7.76.) The anhydrous product was a colourless glass. (Found: C 56.0; H 6.62; OCH_3 14.8. Calc.

for $C_{19}H_{14}O_8$: C 56.1; H 6.59; OCH_3 14.5.) Acetylation of the crystalline racemate or of the anhydrous product yielded a tetraacetate of m. p. 86–88°, identical with the previously [9] described acetate (VIII) of m. p. 84–85°, which obviously was not quite pure. Acetylation of the non-crystallizable residue obtained from the mother-liquors of the crystalline guaiacylglycerol furnished the second tetraacetate (VIII), m. p. 113–114°.

We further want to report the synthesis of the phenolic guaiacylglycerol-β-guaiacyl ether (III). It was carried out in a manner analogous to the preparation of the corresponding methyl ether (II). The reaction of ω-bromo-benzoyl-acetoguaiacone [1] with guaiacol in anhydrous methyl ethyl ketone in the presence of anhydrous potassium carbonate gave ω-(2-methoxy-phenoxy)-benzoyl-acetoguaiacone, which was treated in abs. ethanol with paraformaldehyde and potassium carbonate at room temperature. Reduction of the resulting α - (2-methoxy-phenoxy) - β - hydroxy-propioguaiacone with lithium aluminium hydride yielded the crude substance III as a syrup, from which a crystalline triacetate (IV), m. p. 106–108°, was obtained. (Found: C 62.0; H 6.08; OCH_3 14.06; CH_3CO 28.5. Calc. for $C_{22}H_{26}O_9$: C 61.8; H 5.86; OCH_3 13.90; CH_3CO 28.9.) The acetyl groups were removed from this triacetate by reduction with $LiAlH_4$. The guaiacylglycerol-β-guaiacyl ether (III) thus obtained was a practically colourless syrup. On re-acetylation the triacetate IV was again obtained.

Very recently, Freudenberg [10] has synthesized substance V by a similar procedure and has shown that one of the three dimeric products previously obtained on enzymatic dehydrogenation of coniferyl alcohol [11] is identical with this substance.

The view expressed by Adler and Lindgren [3-5] that guaiacylglycerol-β-aryl ether structures (I) may be essential elements in lignin has thus received further substantial support.

Details regarding the synthesis of III and of related compounds as well as their behaviour in typical lignin reactions, especially in ethanolysis, will be published later.

This work was supported by a grant from *Statens Naturvetenskapliga Forskningsråd.*

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Expansion of Condensed Monolayers of Normal Long Chain Fatty Acids on Admixture of *Iso*-dextropimaric Acid

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Previous investigations [1,2] have shown that myristic and other long chain fatty acids, which give expanded monolayers in pure form, yield condensed monolayers when cholesterol and certain other bulky molecules are added to them. In the following an admixture-effect will be described which is the opposite of the above, *i. e.* an expansion of condensed fatty acid monolayers occurs on addition of a foreign substance, which alone forms a monolayer of the condensed type.

The study was conducted with a continuously recording surface balance of the Wilhelmy-Dervichian type [3]. The fatty acids investigated were palmitic, stearic, arachidic, behenic, lignoceric and myristic acids, of which the last-mentioned forms a monolayer of the expanded type. As the admixture-substance, *iso*dextropimaric acid *, a rosin acid, was employed.

* For a detailed investigation of the monolayer properties of this and other rosin acids, see references 4 and 5.

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* For a detailed investigation of the monolayer properties of this and other rosin acids, see references 4 and 5.

In Fig. 1 are plotted the areas per molecule of monolayers containing different contents of *iso*dextropimaric acid (IdP); these relate to a surface pressure of 1.5 dynes / cm *. The palmitic-IdP layers (Curve *16 C*) are seen to exhibit much higher area values than would be expected if the monolayer behaved as an ideal mixture, *i. e.* if the molecular area of the mixture were given by the expression $A_{mixt.} = x_1A_1 + x_2A_2$ (dotted line). The area increase is also considerable for the stearic-IdP layers (Curve *18 C*), but only relatively slight for the arachidic-IdP layers (Curve *20 C*), and very small for the behenic-IdP layers (curve *22 C*). The areas for the lignoceric-IdP layers follow the ideal curve,

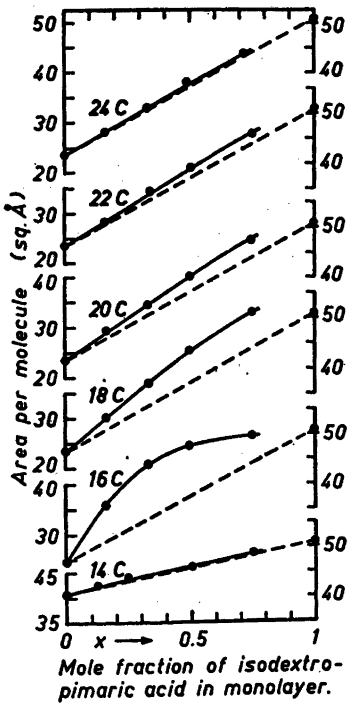

Fig. 1. Molecular areas of normal fatty acid monolayers (C_{14}—C_{24}) containing isodextropimaric acid. *Surface pressure, 1.5 dynes/cm. Dotted lines: calculated molecular areas corresponding to ideal behaviour. Substrate: dilute HCl (pH 3). 20° C.*

* This pressure is frequently employed when defining areas of monolayers of aliphatic long chain compounds.

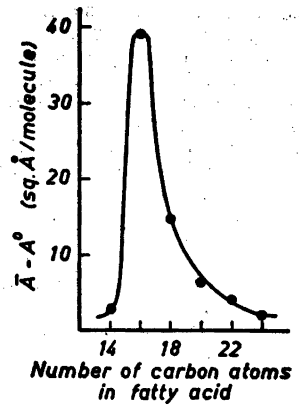

Fig. 2. Expansion of fatty acid monolayers containing isodextropimaric acid *(mole fraction x = 0.1). Ordinate: difference between partial molecular area for* isodextropimaric acid *in the fatty acid monolayer and the molecular area in the pure* isodextropimaric acid *monolayer. Surface pressure, 1.5 dynes/cm. Substrate: dilute HCl (pH 3). 20° C.*

as also do those for the myristic-IdP layers (Curve *14 C*). In Fig. 2 the difference between the actual partial molecular area $\bar{A}$ as determined by the tangent method and the molecular area $A°$ for the pure substance is plotted for *iso*-dextropimaric acid (mole fraction $x = 0.1$) against the number of carbon atoms of the fatty acid forming the other component of the mixture. The different degrees of expansion become clearly evident from the diagram.

Palmitic acid is the first fatty acid with increasing chain length that at room temperature forms a condensed monolayer. It possesses a hydrocarbon chain of sufficient length to give rise to a lateral attraction strong enough to overcome the thermal agitation of the flexible chains. When IdP molecules are added to its monolayer, they force apart the densely packed palmitic acid molecules and owing to their short length (IdP 16 Å, palmitic acid 24 Å) they are able to fill only the lowers parts of the openings formed. The lateral attraction, composed of the attraction between the palmitic and IdP molecules and between the upper ends of the former molecules becomes insufficient to maintain a condensed monolayer. With increasing chain length however, the lateral attraction gradually

increases, until in the case of lignoceric acid, for which the part of the chain projecting above the IdP molecules has a length of about 19 Å, it is sufficient to effect complete condensation even in the presence of IdP molecules.

It may be noted that also in other systems formed by flexible long chain molecules and rosin acid molecules, the relative lengths of the molecules play an important part. In solutions of binary mixtures of rosin acid soap and fatty acid soaps, mixed micelle formation occurs when the fatty acid soaps are sodium myristate and laurate, but not in the case of sodium palmitate and oleate [6].

I wish to express my gratitude to Professor Per Ekwall, Ph. D., the Head of the Institute, for allowing me to use the facilities of the Institute. I am grateful to Professor Einar Stenhagen of the Institute of Medical Chemistry of Uppsala University for samples of the three higher fatty acids and to Dr. C. G. Harris of Hercules Powder Company, Wilmington, USA, for the sample of *iso*dextropimaric acid.

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Self-diffusion and Viscosity in Liquids

B. OTTAR

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In this preliminary communication some problems connected with self-diffusion and viscosity in liquids are treated using a modified kinetic theory. The molecules of a liquid are assumed to move in jumps between equilibrium positions by passing across a potential barrier, $\triangle F$. For a pure liquid in thermodynamic equilibrium, the mole flow from left to right through a unit area normal to the x-direction, is given by

$$\text{Flow}_{x\rightarrow} = v \cdot c/6 \qquad (1)$$

where c is the concentration in moles/cm³. The velocity of the molecules v is equal to $p \cdot \lambda$, where λ is the mean distance between successive equilibrium positions and p is the number of jumps per sec for which an exponential expression should be used. The mole flow may then be written

$$\text{Flow}_{x\rightarrow} = D \cdot c/\lambda \qquad (2)$$

where $D = p\lambda^2/6$ is the coefficient of self-diffusion. In equilibrium the same flow takes place in the opposite direction and the net flow is zero.

If there is a concentration gradient and D is independent of $c(x)$, the net rate of flow becomes

$$\text{net Flow} = \frac{\partial}{\partial c}\,\text{Flow}_{x\rightarrow} \cdot \triangle c = D \cdot \frac{dc}{dx} \qquad (3)$$

since $\triangle c = \lambda \cdot dc/dx$, and Ficks law is obtained.

The diffusion of a group of n arbitrary molecules in the liquid is then considered. The vectorial displacement of the group centroid, when each molecule in the group has made an independent jump, is given by

$$\bar{\lambda}_n = \frac{1}{n}\sum_1^n \bar{\lambda} \qquad (4)$$

The average numerical value of $\bar{\lambda}_n$ for a large group, or a large number of small groups, is

$$\lambda_n = \frac{1}{\sqrt{n}} \cdot \lambda \qquad (5)$$

The flow of such groups in equilibrium (*cf.* eqs. (1) and (2)) is then

$$\text{Flow}_{x\rightarrow} = D_n \cdot c_n/\lambda \text{ with } D_n = \frac{p \cdot \lambda^2}{6\cdot\sqrt{n}} \qquad (6,7)$$

The activation energy of a solute molecule (or ion) in water, is supposed to be due to the formation of hydrogen bonds between this and the water molecules, the solute molecule being able to establish a certain maximum number n_s of hydrogen bonds according to its molecular structure. Only a fraction of these hydrogen bonds are established at any time, and this fraction is continually changing. It is postulated that the diffusion coefficient of the solute molecule is equal to the diffusion coefficient of the surrounding group of n_s water molecules. If Eyring's expression for p is used, the diffusion coefficient becomes

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$$D_{n_s} = \frac{\lambda^2}{6 \cdot \sqrt{n_s}} \cdot \frac{kT}{h} \exp(\triangle F_s/RT) \quad (8)$$

where n_s is the maximum number of hydrogen bonds for a solute molecule, and $\triangle F_s$ is the activation energy of these bonds.

In other liquids n_s may be the coordination number and $\triangle F_s$ the activation energy of exchange of one of the neighbouring solvent molecules. When the $\triangle F_s$ values of two ions are equal, the simpler equation

$$D_{n_1} \cdot \sqrt{n_1} = D_{n_2} \sqrt{n_2} \quad (9)$$

may be used to interpret experimental data.

Conductivity and viscosity. The conductivity of ions at infinite dilution is obtained from eq. (8) by partial differentiation with respect to $\triangle F_s$

$$\text{net Flow} = 2 \cdot \frac{\partial}{\partial \triangle F_s} \text{Flow}_{x \to} \cdot \triangle(\triangle F_s) \quad (10)$$

where

$$\triangle(\triangle F_s) = N \cdot z \cdot e \cdot V \cdot \lambda \quad (11)$$

and Nernst's law is obtained:

$$\Lambda_\infty^{\pm} = D_n \cdot \frac{F^2 \cdot z}{RT} \quad (12)$$

This derivation of Nernst's law assumes that the electric field causes only a differential change in the free energy of activation, this being the foundation of Ohm's law in the present model for a liquid. The importance of this argument lies in the possibility of deriving similar relationship for other properties of a liquid.

The laminar flow in a liquid is proportional to the applied tangential force

$$K = \eta \cdot dv/dz \quad (13)$$

where η is the coefficient of viscosity and dv/dz the velocity gradient normal to the moving layers. The unit of viscosity, the poise, is defined with reference to flow through a centimeter cube, when the top layer moves with a constant velocity v_0 of 1 cm/sec relative to the bottom layer. If this centimeter cube is considered as being divided into molecular layers, and the flow represented as a diffusion of these layers under the influence of an external force K, then the diffusion coefficient of a layer is given by eq. (8) with $n = \frac{1}{2} (N/V_M)^{2/3}$ as only one side of the layer resists the flow, *cf.* eq. (5). The net flow is given by eq. (10) with

$$\triangle(\triangle F) = N \cdot \eta \cdot \lambda \cdot dv/dz \quad (14)$$

From macroscopic considerations the flow is equal to

$$\frac{v_0}{2} \cdot c$$

and the equation

$$\frac{1}{\eta} = \frac{4 \cdot \sqrt{2} \cdot N^{2/3} \cdot V_M^{1/3}}{RT} \cdot D \quad (15)$$

is obtained, where D is the coefficient of self-diffusion for the liquid.

Applications. By assuming a common value of the entropy of activation, eq. (8) yields the following n-values of some simple ions based on $nH_2O = 4$, NH_4^+ 11.6; Cl^- 12.1; K^+ 11.9; Na^+ 15.0; Ag^+ 11.2; NO_3^- 21.7; Ac^- 18.8; Ox^{2-} 37.0; Ca^{2+} 33.9; Ba^{2+} 33.9; SO_4^{2-} 29.1;

Values of $\Lambda\infty$ taken from Handbook of Chem. and Physics. Log D/T plotted against $(1/RT)^2$ over the temp. range 50—156° C.

Possible ionic structures are $K^+ \cdot 4\,H_2O$, $NO_3^- \cdot 6\,H_2O$, $SO_4^{2-} \cdot 10\,H_2O$. Ca^{2+} appears to have a double layer of H_2O molecules.

Comparison of the diffusion coefficients of silicate [1], phosphate [2], vanadate [1] and chromate ions [3], with the corresponding Me_2O_7-ions gives n_3/n_1-values close to 1.50 which agrees well with a number of 4 or 6 oxygen-atoms, respectively, interacting with the solvent molecules. The corresponding values $n_3/n_1 = 1.99$ and $n_4/n_1 = 2.56$ for the more complex vanadate ions indicate the open tetrahedral structures V_3O_{10} and V_4O_{12}.

Coefficients of self-diffusion calculated from viscosity data using eq. (15) agree with experimental coefficients of self-diffusion for the following liquids, water [4], heavy water [4], mercury [5], ethyl bromide [6] and benzene [6]; the calculated values of alcohols [7] are lower due to the effect of deuterium on the hydrogen bonds in these liquids when an alcohol-d is used as tracer. This effect is large in methyl and ethyl alcohol, small in *n*- and *t*-propyl alcohol and almost disappears in *n*- and *t*-butyl alcohol.

A more detailed report on these matters will be given elsewhere.

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Desoxypodophyllotoxin, Isolated from Podophyllin

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In the course of a systematic re-investigation of the constituents of Podophyllin (the resin from *Podophyllum peltatum* L.) by means of chromatographic methods [1] a substance has been isolated as colourless needles, m. p. 170–172° C corr., yield approximately 0.1 %. It analyzed as $C_{22}H_{22}O_7$. (Found: C 65.7; H 5.58; OCH_3 23.3. Calc. C 66.3; H 5.57; 3 OCH_3 23.4.) The optical rotation in chloroform solution (c = 0.98) was $[\alpha]_D^{22} = -123° \pm 10°$. These data are close to those reported for desoxypodophyllotoxin (I), a compound which was prepared artificially by Hartwell, Schrecker and Johnson [2] by hydrogenolysis of podophyllotoxin chloride (III). These authors reported a melting point of 168–69° and $[\alpha]_D^{20} = -115°$ (chloroform, c = 0.50). The location of our natural substance on paper chromatograms (formamide-paper/benzene [1]) close to the solvent front and thus with a much higher R_F-value than podophyllotoxin (II) is consistent with the absence of the hydroxy-group (*cf.* Ref. [1], p. 950). Further evidence is provided by the ultra-violet spectrum, Fig. 1. It shows the essential features of the spectrum reported by Schrecker and Hartwell [2] for the synthetic desoxypodophyllotoxin. Although the minimum is slightly flattened, this and the maximum are identical with those of the synthetic substance with respect to the wave lengths. Finally on base-catalyzed epimerization the natural product yielded a substance identical with desoxypicropodophyllin (I, *cis*-2 : 3-*trans*-3 : 4) by melting point and optical rotation (Found m. p. 171–173°C, $[\alpha]_D^{21} = +33° \pm 10°$ (c = 0.25, chloroform); lit. m. p. 170.7–172.0°, $[\alpha]_D^{21} = +32°$ (c = 0.50, chloroform) [2]; m. p. 168–170°, $[\alpha]_D^{19} = +39°$ (c = 0.53, chloroform) [3, p. 5922]; m. p. 169.5–171°, $[\alpha]_D^{20} = +37°$ (c = 1.0, chloroform) [3, p. 5922].

To our knowledge this is the first time desoxypodophyllotoxin has been isolated from a *Podophyllum* species. It has previously been obtained from *Juniperus*

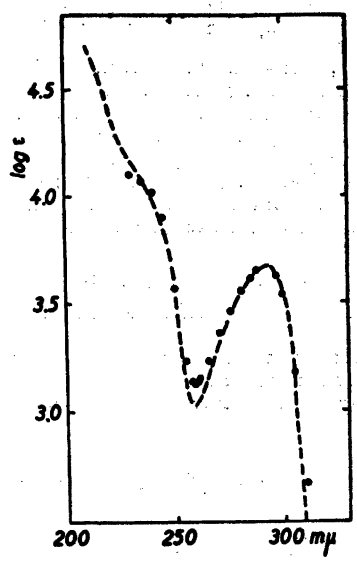
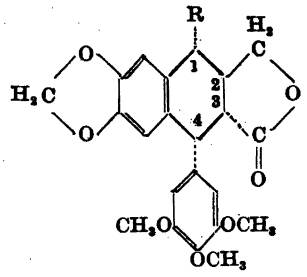


Fig. 1. Absorption in the ultra-violet of desoxypodophyllotoxin (I) in 95 % ethanol. ..... Curve given by Schrecker and Hartwell [2] for their synthetic product. O Natural product described in the present communication.

silicicola (Small) Bailey by Hartwell and co-workers [2,4], and was originally named silicicolin (m. p. 173.9–175.5° corr., $[\alpha]_D^{19} = -119$ (chloroform) (Ref.[4], *cf.* Ref.[2], footnote 15); m. p. 171–172° corr.[2]. The discoverers of silicicolin also demonstrated [7] that anthricin, isolated in 1940 (Noguchi and Kawanami [5]) from *Anthriscus silvestris* Hoffm. and hernandion, isolated in 1942 (Hata [6]) from *Hernandia ovigera* L. were in fact identical with desoxypodophyllotoxin.

A direct detection of desoxypodophyllotoxin in extracts of fresh plant material is impracticable even with the highly sensitive paper chromatographic technique [1]. It has not been definitely proved, therefore, whether the isolated desoxypodophyllotoxin is a genuine constituent in the plant or an artifact. However, a formation of the substance by dehydroxylation of podophyllotoxin, the principal constituent of podophyllin, or in fact of any other recorded constituent, must be regarded as extremely unlikely under the conditions normally applied during the preparation of

the commercial resin from the plant material.



I R = H desoxypodophyllotoxin $C_{22}H_{22}O_7$ (398.4) *trans*-2:3-*cis*-3:4
II R = OH podophyllotoxin $C_{22}H_{22}O_8$ (414.4) *trans*-1:2-*trans*-2:3 *cis*-3:4
III R = Cl podophyllotoxin chloride *(Hartwell and co-workers)*

Analyses are by Mr. P. Hansen, Universitetets kemiske laboratorium København. This work is part of investigations suppported by *Statens almindelige Videnskabsfond.*

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A Note on the Melting Point of Podophyllotoxin

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The melting points previously reported in the literature for podophyllotoxin vary within the limits *ca.* 100—189° C, the majority falling in the ranges 110—118, 158—162 and 179—184° C. The explanation of this divergency is (1) that the substance is polymorphic, and occasionally (2) that investigators have overlooked [7], *cf.* [8] the fact that podophyllotoxin retains liquid of crystallization very strongly [5,8], so that some melting points actually refer to solvated products. In either case the experimental melting point will be dependent upon the type of apparatus, the rate of heating *etc.*

The melting point 188—189° C, recently communicated by us [1] is the highest ever obtained and it may need a few comments.

The general experience accumulated in this laboratory concerning the melting points of various podophyllotoxin preparations is summarized below. Unless otherwise stated the melting points — or rather melting intervals — were determined with the hot-stage microscope, essentially according to Kofler [8]. The apparatus was carefully calibrated against a series of pure standard substances. All podophyllotoxin samples were obtained in this laboratory by chromatographic fractionation of Podophyllin, *Pharmacopoia Danica Ed. IX*, on either alumina or formamide-impregnated silica-gel, benzene being the eluent.

Several preparations of podophyllotoxin, recrystallized from commercial wet benzene and dried in the atmosphere under an infra-lamp (*i. e.* exposed to a maximum temperature of 50° C) had a melting point of 110—115°. The same melting point was normally obtained in a closed capillary heated in a silicone bath. Elementary analysis and the percentage loss upon drying to constant weight at 0.01 mm Hg (continuous pumping) and 100° C (up to 18 hours) almost invariably indicated the composition 2 $C_{22}H_{22}O_8$, 2 H_2O, C_6H_6 of the original air-dried solvate, *cf.* Ref. [4,5].

The pure, unsolvated podophyllotoxin obtained by the above drying procedure in the first period of our work had a m. p. 183—84°. Later preparations, however, obtained without any deliberate alteration of procedure, melted completely at 111—118°. When heated at 150° for 1—2 hours on the microscope slide the substance resolidified and the crystals eventually melted at 183—184°. In a few cases, particularly with excessively slow heating it was possible to pass through the temperature range 111—118° without any visible phase-transition. Then the substance finally melted at 188—189°. Such was the case with the sample mentioned in our first paper [1].

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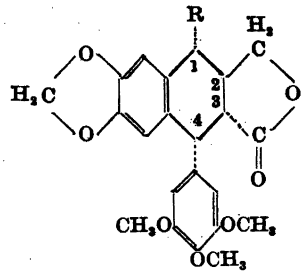

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Recently we have obtained a product, which, recrystallized from benzene and dried to constant weight under the conditions specified above, had a single melting point at 188—189°, both with the Kofler apparatus and as determined by the capillary method (rate of heating in the melting region 2°/min). Only a very slight temporary moistening was noticeable at about 115° C, even when heating rapidly. When, beyond the melting point, the heating was discontinued and the hot-stage allowed to cool to 180° the substance crystallized, and repeated melting point determination now gave 187—188°.

The molecular identity of the new modification with podophyllotoxin m. p. 183—184° was proved by elementary analysis (Found: C 63.29; H 5.55). Calc. for $C_{22}H_{22}O_8$: C 63.75; H 5.35), by paper chromatography (*vide* Ref.[1]), by its ultraviolet absorption spectrum and by the optical rotation. Finally on epimerization with sodium acetate [5] it yielded picropodophyllin, m. p. 228—231° (lit.[6] 231.5—232.5°) which did not depress the melting point of an authentic sample.

Besides the modifications already mentioned a fourth one is known, melting at 158° (Ref.[5]). This modification has never been encountered in this laboratory. Its existence has, however, recently been confirmed by American investigators [8] who report a melting point of 161—162° C.

It appears, therefore, that according to circumstances, dried and unsolvated podophyllotoxin may come out in four different crystal modifications

I m. p. 114—118 (without foaming, Ref.[4]); 111—118 (this laboratory)
II m. p. 161—162 (Ref.[3]); 158 (without foaming, Ref.[5]); 157 (Ref.[10])
III m. p. 183—184 (Ref.[8], p. 2913 and the present authors); 179 (Ref.[11])
IV m. p. 188—189 (the present paper)

Various air-dried solvates have been described in the literature,

e. g., 2 PT, 2 H_2O, C_2H_5OH, m. p. 106—108 (Ref.[4])
2 PT, 2 H_2O, C_6H_6, m. p. 114—116, foaming (Ref.[9] and Ref.[6], footnote 32); m. p. 114—117 (Ref.[7], *cf.* Ref.[8]); 110—115 (this laboratory).

Since these solvates melt in much the same region as modification I, the postulated existence of I is open to the criticism that the experimental observations (*vide infra*) might as well be explained by assuming that the "modification" is a partially solvated podophyllotoxin melting around 115°, then loosing solvent of crystallization, the last trace of which disappears at about 150°, subsequently resolidifying as modification III or IV, which finally melts at the appropriate temperature. Evidence against this explanation is, however, that both Borsche and Niemann's and our own preparation of modification I was dried under carefully controlled conditions and analyzed as unsolvated podophyllotoxin. No loss of weight was observed on further drying at 125°, 0.01 mm Hg for two hours. It should also be mentioned that the melting process of I was not accompanied by the foaming, characteristic of the solvates.

Our thanks are due to Dr. A. W. Schrecker for discussion and for permission to publish the melting point of modification II. Analyses are by Mr. W. Egger, Chemical Laboratory, University of Copenhagen. This work is part of investigations supported by *Statens almindelige Videnskabsfond.*

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The Determination of Oxime Nitrogen

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The acid hydrolysis method which is usually employed for oxime determination does not give quantitative results as reported by Novak and Wilson [1]. One cause of error is that the reaction is reversible and the hydroxylamine formed recombines with the keto compound. Moreover, this method does not give satisfactory results in the presence of ascorbic acid [2]. Yamafuji *et al.*[3] developed a quantitative method for oxime determination under alkaline conditions. This method is suitable for oximes of some keto compounds. It is, however, not applicable to acetoxime or glucosoxime. I, therefore, developed a modification of the acid hydrolysis method involving the addition of 2,4-dinitrophenylhydrazine and can confirm that this procedure gives good results with various oximes even in the presence of interfering substances.

The determinations were carried out according to the following procedure.

Reagents: 2 % 2,4-dinitrophenylhydrazine in 6 *N* H_2SO_4, 10 % (v/V) ethyl acetate in benzene and benzene and hydroxylamine reagents, prepared according to Csáky's method [4], *i. e.* 35 % Na-acetate, sulphanilic acid (30 % acetic acid solution), I_2 in glacial acetic acid, α-naphthylamine (30 % acetic acid solution) and Na-arsenate.

Conditions of hydrolysis: 1 ml of 2,4-dinitrophenylhydrazine in 6 *N* H_2SO_4 and 2 ml of the sample (containing 0.1 ~ 0.4 μmole of oxime) were mixed and boiled for 2 hours on a water bath.

Extraction of 2,4-dinitrophenylhydrazine and hydrazone: After cooling the appropriate amount of water was added to the hydrolyzed mixture *. Usually the total volume was 7 ml. Hydrazone together with hydrazine were extracted three times with 3 ml of 10 % ethyl acetate (in benzene) and then twice with 3 ml of benzene. After separating the water phase from the benzene 5 ml of the water phase was transferred to another test tube and mixed with 2 ml of Na-acetate solution to neutralize excess H_2SO_4 and then extracted again with 3 ml benzene three times. The 10 % ethyl acetate was used because it had a stronger extracting capacity than benzene only for 2,4-dinitrophenylhydrazine and especially the hydrazone. However, it also extracts free hydroxylamine in the absence of 2,4-dinitrophenylhydrazine as shown in Table 1. It is not, therefore, desirable to use this solvent more than is necessary.

Table 1. Effect of extraction with 10 % ethyl acetate in the absence of 2,4-dinitrohydrazine.

Number of extractions		Extinction
in acid phase with 10 % Et—Ac	in alkaline phase with benzene	
0	0	38.3
1	3	35.6
3	3	32.2
5	3	27.8
0	0	38.0
1	4	39.7
3	3	38.7
5	3	36.8

Colourimetry: 5 ml of the water phase thus obtained was taken out as the aliquot for hydroxylamine determination according to Csáky and measured with Fischer's photoelectric colourimeter using 525 mμ filter. A solution, treated similarly to the test solutions without oxime, was used as reference solution.

The experimental results obtained with several oximes are shown in Table 2. The standard solutions of pyruvic oxime and glucosoxime were prepared from solid samples and those of α-ketoglutaric oxime and acetoxime were obtained by diluting a mixture of 1 part of 4×10^{-4} *M* hydroxylamine and 1 part of the corresponding keto compounds (0.2 mg/ml) after a reaction time of 3 hours. The recovery in using this method was always more than 95 %. With the alkaline method, however, pyruvic oxime and α-ketoglutaric oxime are determined quantitatively. As the latter method is unsuitable for acetoxime and glucosoxime the new method may be said to have a wider application, being at the

* The suitable amount depends on the kind of colourimeter.

Table 2. Recovery of hydroxylamine from various oximes.

Oxime	Added conc. ($10^{-7}M$)	Found conc. ($10^{-7}M$)	Recovery %
Pyruvic-oxime	3.98	3.76	94
	3.64	3.57	98
	2.99	2.92	98
	2.73	2.53	93
	1.99	1.88	95
	1.82	1.78	98
	1.00	1.02	102
	0.91	0.90	99
Glucos-oxime	4.40	4.34	99
	2.20	2.26	103
α-Ketoglutaric oxime	4.14	3.96	95
	2.07	1.88	95
Acetoxime	4.14	4.06	98

Table 3. Effect of coexisting substances on the pyruvic oxime determination. Amount of oxime: 1.8 × 10^{-7}mole.

Coexisting substance		2,4-Dinitrophenyl-hydrazine ml 2 % solution	Extinction
Substance	Amount		
None		1	34.5
Pyruvate	0.2 mg	1	34.5
»	1 mg	1	30.8
»	1 mg	2	34.5
α-Ketoglutarate	0.2 mg	1	34.5
»	1 mg	1	33.7
»	1 mg	2	34.5
Ascorbic acid	0.2 mg	2	34.5

Table 4. Effect of nitrite and hydroxylamine. Nitrite was decomposed by adding a sufficient amount of urea.

Added (10^{-7}mole)			Found NO_2(10^{-7}mole) after I_2-oxidation (total NH_2OH)	Found NH_2OH / (NH_2OH + oxime) %
NO_2	NH_2OH	Pyruvic oxime		
10	0	1.82	1.74	95
1.0	1.0	1.99	2.91	97
1.0	1.0	0.99	1.97	99
0.5	0.5	0.80	1.34	103

same time more accurate than the ordinary acidic methods.

When oximes of ketoacids were hydrolysed ordinarily in an acidic medium, the liberated hydroxylamine was decomposed by various substances, including the liberated ketoacids. This interfering effect was eliminated by addition of 2,4-dinitrophenylhydrazine as well as by using the alkaline method (Table 3).

As the coexisting nitrite tends to decompose hydroxylamine and is colourized simultaneously, it is desirable to remove the nitrite beforehand. This was achieved when the test solution containing nitrite was kept for 20 min. with urea in an acidic solution before addition of 2,4-dinitrophenylhydrazine. In this case, it was necessary to increase the amount of Na-acetate to neutralize excess of acid. The results are shown in Table 4. This method is more convenient because oxime can be determined independently.

The author is greatly indebted to Professor Artturi I. Virtanen for his kind advice and encouragement.

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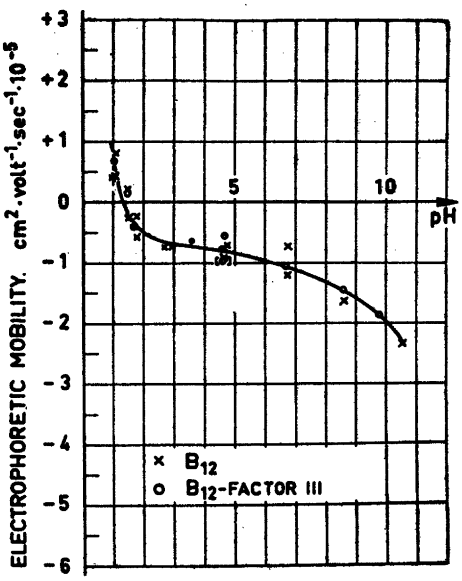

Fig. 1.

Electrophoretic Studies of Vitamin B_{12}-factors. III

H. NIHLÉN and L.-E. ERICSON

LKB Research Laboratory, Äppelviken, Sweden; Division of Food Chemistry, Royal Institute of Technology, Stockholm, Sweden

Bernhauer *et al.* have recently isolated five different vitamin B_{12}-factors from digested sewage sludge [1,2]. One of these factors, provisionally called "Factor III", has practically the same microbiological activity towards *E. coli* and *Lb. leichmannii* in the tube-assay as cyanocobalamin (vitamin B_{12}) and has strong activity towards *Ochromonas malhamensis*. The antipernicious anemia activity of Factor III is similar to that of cyanocobalamin [3]. Factor III, however, differs from other vitamin B_{12}-factors with regard to R_F-values and distribution coefficients [3]. The absorption spectrum of Factor III closely resembles that of cyanocobalamin with the difference that the narrow band at 278 mμ is only faintly marked while a wide band at 295 mμ appears. Such a band has never been noticed before in any other vitamin B_{12}-factor. Factor III like cyanocobalamin itself is able to form aquo- and hydroxo-complexes as well as a cyano-complex containing more than one CN-group per molecule of Factor III.

A comparison between the effect of pH on the electrophoretic mobility of Factor III and of cyanocobalamin has been carried out in our laboratories.[4] It was concluded at first that the electrophoretic properties of Factor III closely resembled those of cyanocobalamin, except at pH values between 1 and 2. On reinvestigating thoroughly the electrophoretic mobility of cyanocobalamin in this pH-region, we found that the isoelectric point of this compound is somewhat lower than pH 1.9, as was first stated, and occurs at pH 1.5. Factor III has its isoelectric point at the same pH-value. The variation of

same time more accurate than the ordinary acidic methods.

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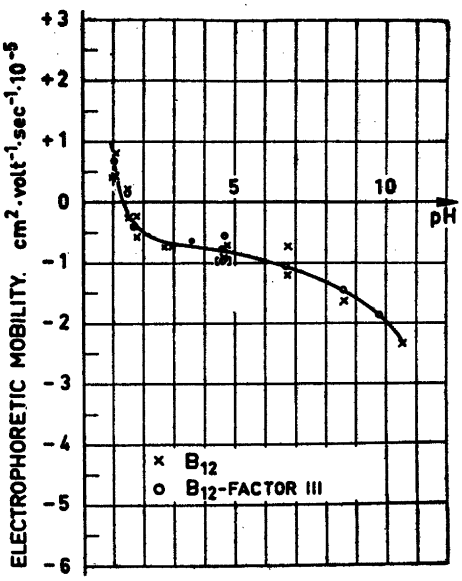

Fig. 1.

Electrophoretic Studies of Vitamin B_{12}-factors. III

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Bernhauer *et al.* have recently isolated five different vitamin B_{12}-factors from digested sewage sludge [1,2]. One of these factors, provisionally called "Factor III", has practically the same microbiological activity towards *E. coli* and *Lb. leichmannii* in the tube-assay as cyanocobalamin (vitamin B_{12}) and has strong activity towards *Ochromonas malhamensis*. The antipernicious anemia activity of Factor III is similar to that of cyanocobalamin [3]. Factor III, however, differs from other vitamin B_{12}-factors with regard to R_F-values and distribution coefficients [3]. The absorption spectrum of Factor III closely resembles that of cyanocobalamin with the difference that the narrow band at 278 mμ is only faintly marked while a wide band at 295 mμ appears. Such a band has never been noticed before in any other vitamin B_{12}-factor. Factor III like cyanocobalamin itself is able to form aquo- and hydroxo-complexes as well as a cyano-complex containing more than one CN-group per molecule of Factor III.

A comparison between the effect of pH on the electrophoretic mobility of Factor III and of cyanocobalamin has been carried out in our laboratories.[4] It was concluded at first that the electrophoretic properties of Factor III closely resembled those of cyanocobalamin, except at pH values between 1 and 2. On reinvestigating thoroughly the electrophoretic mobility of cyanocobalamin in this pH-region, we found that the isoelectric point of this compound is somewhat lower than pH 1.9, as was first stated, and occurs at pH 1.5. Factor III has its isoelectric point at the same pH-value. The variation of

electrophoretic mobility with pH for both Factor III and cyanocobalmin is shown in Fig. 1. It can be seen that the electrophoretic mobility-pH-curves of these two compounds are for all practical purposes identical.

The authors wish to express their thanks to Professor K. Bernhauer, Head of the Research Laboratory of Aschaffenburger Zellstoffwerke A. G., Stockstadt, Germany, for supplying us with Factor III.

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Study of a Peptic Degradation Product of Cytochrome c

I. Purification and Chemical Composition

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A ferriporphyrin c-peptide obtained from *beef* cytochrome c by digestion with pepsin and previously described by Tsou [1,2] has been purified by a partition chromatographic method.

The peptide component of this haemopeptide has been shown to be composed of eleven amino acid residues arranged in the following sequence: Val-Glu(NH_2)-Lys-CySH-Ala-Glu(NH_2)-CySH-His-Thr-Val-Glu. The two cysteine residues occurring in this chain are linked to the side-chains 2 and 4 of the ferriporphyrin prosthetic group *via* two thioether bridges.

The sequence of amino acid residues in the ferriporphyrin c-peptide prepared by peptic degradation of *salmon* cytochrome c is identical with the one found in the split product of beef cytochrome c. The corresponding peptide derived from *chicken* cytochrome c, however, differs from the others by the replacement of the alanine residue by a serine residue.

Through digestion of horse cytochrome c with *pepsin*, Tsou [1] has obtained a brown-red coloured split product of relatively low molecular weight. This "pepsin-modified cytochrome c" contained the prosthetic group of the enzyme linked to a peptide component which represented a part of the original protein moiety of cytochrome c. The haemopeptide exhibited interesting enzymatic properties [2] different from those of native cytochrome c. Tsou's degradation method was recently applied to cytochromes of three different animal species [3]; the peptic split products of beef, chicken and salmon cytochromes c were prepared and found to show a high degree of similarity.

In another recent investigation[4] a *tryptic* degradation product of cytochrome c was purified and its chemical structure determined. The peptide component was shown to contain nine amino acid residues arranged in the following sequence *:

CySH—Ala—Glu(NH_2)—CySH—His—Thr—Val—Glu—Lys.

* Throughout this paper the abbreviations for the amino acid residues suggested by Brand and Edsall [5] will be used. The symbol for cysteic acid will be $CySO_3H$. The arrangement of amino acid residues in peptides will be described as suggested by Sanger [6].

The two cysteine residues occurring in this chain are linked to the side chains 2 and 4 of haematohaemin c[7] *via* two thio-ether bridges[8, 9]. The same sequence of nine amino acid residues could be demonstrated to occur in the ferriporphyrin c-peptides obtained by tryptic degradation of three different mammalian cytochromes[10].

In the present investigation the *peptic* degradation product, which Tsou had thought to be a pure substance, has been prepared free from contaminating peptides, and the chemical structure of the purified product has been established. By comparing the sequence of amino acid residues in the haemopeptides isolated from peptic digests of beef, chicken and salmon cytochromes c, an unexpected instance of species specificity of protein structure has been found.

MATERIAL

Cytochrome c. Beef, chicken * and salmon cytochromes were prepared from minced hearts by the method of Keilin and Hartree[11]. In the case of salmon, however, the initial extraction of the enzyme from the mince was carried out using dilute sulphuric acid[3] instead of trichloroacetic acid. After fractionation of the extracts with ammonium sulphate and precipitation of the enzyme with trichloroacetic acid, the cytochromes were dissolved in dilute ammonia, dialyzed against 0.05 *N* ammonia and finally against distilled water.

Beef cytochrome c was further purified as described by Paléus and Neilands[12]. For chicken and salmon cytochromes the method of Margoliash[13] was followed. In all three cases Amberlite XE-64 was the ion exchange resin used. The preparations of cytochrome c thus obtained had iron contents of 0.41 % (beef), 0.40 % (chicken) and 0.31 % (salmon).

Proteolytic enzymes. The samples of pepsin and of trypsin used in this work were crystallized products of the "Bios Laboratories". Their proteolytic activities were tested by Anson's haemoglobin method[14] and found to be, respectively, 0.0266 units/mg and 0.0065 units/mg. The crystallised *Bacillus subtilis* proteinase "subtilisin"[15, 16] was kindly made available to us by Professor K. Linderstrøm-Lang and Ing. M. Ottesen.

METHODS

Preparation of the peptic split product of beef cytochrome c. For the splitting of cytochrome c with pepsin the procedure of Tsou[1] was adopted. However, in contrast to his experience, after digestion in acid solution and neutralisation of the digest, the degraded pigment could not be precipitated quantitatively by making the solution 85 % saturated with ammonium sulphate. This indicated incomplete splitting. The incompletely digested material had to be subjected to another incubation with pepsin **. In the following a representative two-stage digestion experiment is described in detail.

A solution of 0.830 g beef cytochrome c in 83 ml distilled water was mixed with 20 ml 0.3 *N* HCl. To the acid solution (pH 1.55) was added 16.1 mg pepsin dissolved in a few ml of 0.01 *N* HCl. The mixture was incubated 28 h at 25° C and the digestion stopped by adding 20 ml 0.3 *N* NaOH. Solid ammonium sulphate was added to the slightly alkaline solution (pH 8.5) to make it 85 % saturated. Most of the pigment was precipitated, but on filtration the solution also proved to be somewhat coloured. The filtrate was discarded. The precipitate was dissolved from the filter using 0.01 *N* HCl and the pH of the

* We wish to thank AB. Findus, Hälsingborg, for kindly supplying frozen chicken hearts.

** The incomplete digestion of cytochrome samples appears to have been caused by the presence of some substance inhibiting the peptic proteolysis. The inhibitor is likely to have its origin in the ion exchange resin used for the purification of the cytochromes. The inhibitor can be removed from the cytochrome preparations by reprecipitating them with trichloroacetic acid from ammonium sulphate solution. Inhibitor-free cytochrome preparations do not require the two-step digestion with pepsin described above.

solution adjusted to pH 1.60 with 0.3 *N* HCl. To the acid solution (124 ml) 9.8 mg pepsin were added. After 20 h at 28° C the digestion mixture was neutralized by the addition of 0.3 *N* NaOH and made 85 % saturated with ammonium sulphate. Now the precipitation of the pigment was complete. The precipitate was collected by filtration and dissolved from the filter with 100 ml water containing a few drops of ammonia. The dark red solution was mixed with a solution of 280 g ammonium sulphate in 500 ml water and the pigment precipitated by the addition of 19 ml of 20 % aqueous trichloroacetic acid. The precipitate was centrifuged down and dissolved in the smallest possible amount of 0.05 *M* NH_3. The ammoniacal solution was dialyzed in the cold against 0.01 *M* phthalate buffer of pH 5.0 (three changes) and finally against distilled water (two changes). This resulted in an almost complete precipitation of the pigment. The brown-red powdery material was dissolved in 0.02 *M* NH_3 and the precipitations by ammonium sulphate and trichloroacetic acid repeated. Following dialysis against phthalate buffer and distilled water, the peptic degradation product of cytochrome c was collected by centrifugation and dried. Yield: 101 mg.

Purification of the ferriporphyrin c-peptide by partition chromatography. The final purification of the peptide was achieved using essentially the same method as that previously described for the tryptic split product of cytochrome c[4]. To 15 g of carefully washed and dried Hyflo Supercel was added 5.5 g of the lower layer of a freshly prepared mixture of *n*-butanol, glacial acetic acid and water (4 : 1 : 5). After thorough mixing, the Hyflo Supercel was suspended in a suitable volume of the upper phase of the solvent system so as to form a slurry and was poured into a chromatography tube 1.9 cm in diameter. After settling, the Hyflo Supercel formed a column 18.5 cm high. On the top of this column was placed 74.5 mg of the ferriporphyrin c-peptide dissolved in the smallest possible volume of the lighter phase. The chromatogram was developed with the help of larger amounts of the upper layer of the solvent mixture. In order to prevent the pigment from being partially destroyed, the ferriporphyrin c-peptide during chromatography had to be protected from direct illumination. The main component formed a dark brown band nicely moving down the column, the R_F value being approximately 0.9 in the beginning and somewhat decreasing in the course of the development. The main band was closely preceded by a lighter brown-coloured fraction and followed by a greenish band. Sometimes another brown-coloured band travelling slowly was observed. The effluent containing the main fraction was collected separately and evaporated to dryness *in vacuo* in a desiccator over conc. H_2SO_4 and solid KOH. The dried material was dissolved in as little 0.05 *M* ammonia as possible and dialysed in the cold against several changes of 0.01 *M* phthalate buffer pH 5 and subsequently against distilled water. The precipitated pure ferriporphyrin c-peptide was centrifuged down and dried. The final yield was 41 mg.

Table 1. Amino acid composition of the peptic degradation products of three different cytochromes.

Ferriporphyrin c-peptide preparation		Amino acids found in the hydrolysate					
Beef	A	Glu	Thr	Ala	His	Val	Lys
	B	2.99	1.01	1.00	1.11	2.09	1.12
Salmon	A	Glu	Thr	Ala	His	Val	Lys
Chicken	A	Glu	Thr	Ser	His	Val	Lys

A: Amino acids determined by qualitative paper chromatography.
B: Moles of amino acid found, using the method of Moore and Stein [17, 18].

Determination of the constituent amino acids. For a qualitative determination 0.4 mg of the purified preparation were hydrolyzed overnight in a sealed capillary with 5.7 *N* HCl at 105° C, and the hydrolysate subjected to two-dimensional chromatography on Whatman No. 4 filter paper using phenol/NH_3/HCN in the first and collidine/lutidine/water in the second direction. For a quantitative determination 1 mg of the ferriporphyrin c-peptide was hydrolysed and the resulting amino acid mixture analysed by a modification [17] of the method of Moore and Stein [18]. The results of these determinations are summarised in Table 1.

Determination of the amino-terminal amino acid residues. The method used was Sanger's DNP technique [19]. 2.25 mg of the ferriporphyrin c-peptide were reacted with fluorodinitrobenzene in aqueous ethanol containing sodium bicarbonate. After 10 hrs. standing at room temperature with occasional shaking, the reaction mixture was diluted with water and extracted three times with peroxide-free ether. On acidification with HCl, the DNP derivative of the ferriporphyrin c-peptide precipitated quantitatively in the form of a brown powder. It was centrifuged down, washed with water, dried, and finally washed with ether.

0.5 mg of the DNP-derivative was hydrolysed with 5.7 *N* HCl at 105° in a sealed capillary. After 7 hrs. heating the yellow solution, in which black grainy material derived from the porphyrin moiety was floating, was diluted with water and extracted with ether. Paper chromatography of the aqueous solution with the butanol-acetic acid solvent of Partridge [20] revealed the presence of ε-DNP-lysine and im-DNP-histidine, besides free amino acids. In the ethereal extract DNP-valine was identified by the paper chromatographic methods of Biserte and Osteux [21] and of Blackburn and Lowther [22]. This DNP amino acid was further characterized by eluting it from the paper after chromatographic separation and heating it with concentrated ammonia solution [23] in a sealed capillary; after 12 hrs. at 108° a considerable part of the DNP-derivative had been split and free valine could be identified by paper chromatography.

On the paper chromatograms of the ether-soluble fraction a slow-moving and considerably weaker yellow spot (R_F 0.13 in phenol-*iso*amyl alcohol-water solvent [21]) was also found, besides the strong spot of DNP-valine (R_F 0.75). This substance was obtained as the main ether-soluble product, when the DNP-derivative of the ferriporphyrin c-peptide was dissolved in a mixture of equal parts of glacial acetic acid and concentrated HCl and hydrolysed at 108° for 1/2 h only. On further hydrolysis with 5.7 *N* HCl (12 hrs. at 108°) this yellow product gave rise to DNP-valine and free glutamic acid and was thus shown to be DNP-Val-Glu.

Splitting with trypsin. 1.0 mg of the peptic split product of bovine cytochrome c was dissolved in 0.50 ml of a 0.005 *M* ammonia solution, mixed with 0.05 ml 0.2% aqueous trypsin solution and incubated 23 hrs. at 36° C. The digest was then heated 5 minutes in a boiling water bath to inactivate the trypsin and was evaporated to dryness. The dry brown material was dissolved in a small volume of dilute ammonia and the solution placed on a strip of Whatman No. 4 paper in the form of a line 9 cm long. The chromatogram was developed one-dimensionally using butanol-acetic acid [20] as the solvent. The pigment moved down the paper in the form of a broad diffuse brown band (R_F 0.22—0.45). After drying of the chromatogram, a narrow strip was cut off from its long edge and sprayed with ninhydrin; on heating a strong violet colour developed at R_F 0.13. The ninhydrin-positive substance was eluted from the untreated part of the chromatogram.

One third of the eluted material was hydrolysed with 5.7 *N* HCl overnight at 105°. In the hydrolysate 3 amino acids were identified by paper chromatography: valine, glutamic acid and lysine. One third of the peptide was investigated using the DNP technique [6]; the dinitrophenylated peptide, on hydrolysis with 5.7 *N* HCl (10 hrs. at 105°), yielded DNP-valine, ε-DNP-lysine and glutamic acid. The remainder of the eluted peptide was subjected to electrophoresis using the method previously described by the senior author [24] ("Munktell 20, 150 g" filter paper; *M*/15 phosphate buffer pH 6.4; 75 volts; 2.5 mA; 5 hrs.) and it was found to migrate towards the cathode.

Preparation of the porphyrin-free peptide component of the peptic split product of cytochrome c. The thio-ether bridges were split by the method of Paul [25] following the procedure previously described for the removal of the pigment moiety from the tryptic degradation product of cytochrome c[4]. The thiol groups set free through the action of silver salts were oxidized to sulphonic acid groups by treatment with performic acid [26].

12 mg of the peptic split product of cytochrome c were dissolved in 5 ml of 60 % acetic acid, mixed with a warm solution of 80 mg silver sulphate in 10 ml water and heated at 50° for 9 hrs. Then the solution was evaporated to dryness *in vacuo* at low temperature. To the residue a mixture of 3 ml 87 % formic acid and 0.3 ml 30 % hydrogen peroxide was added. After 20 minutes standing at room temperature the reaction mixture, which had turned light yellow through oxidative degradation of the porphyrin, was diluted with water and evaporated to dryness *in vacuo* at a temperature not exceeding 30° C. Addition of water and evaporation to dryness was repeated twice in order to remove all the hydrogen peroxide. The residue was dissolved in 40 ml distilled water of 60°, 2 drops of 20 % H_2SO_4 added and H_2S passed into the warm solution. The precipitate of Ag_2S was removed by filtration and the clear filtrate reduced to a volume of about 10 ml by careful evaporation *in vacuo* at a temperature not higher than 25°. The sulphuric acid was removed by extracting three times with a solution of 0.25 ml tri-*n*-octylamine in 10 ml chloroform [27, 28], followed by chloroform and ether. The aqueous solution was evaporated to dryness leaving behind the porphyrin-free peptide.

A small amount of the peptide was heated 20 hrs. with 5.7 *N* HCl at 105°; in the hydrolysate the following amino acids were found by paper chromatography: $CySO_3H$, Glu, Thr, Ala, Val, His and Lys.

Partial acid hydrolysis of the porphyrin-free peptide. Separation and examination of the split products. The peptide prepared from 12 mg of bovine ferriporphyrin c-peptide as described above was hydrolysed 4 days at 37° C in concentrated HCl. After removal of excess HCl, the hydrolysate was adjusted to pH 5 and fractionated in the four-compartment ionophoresis cell described by Sanger and Tuppy [29]. The peptide and amino acid mixtures present in the acidic and neutral fractions were investigated by two-dimensional paper chromatography using essentially the same methods as those reported by Sanger and coworkers [29, 30].

Digestion of the porphyrin-free peptide with subtilisin. Porphyrin-free peptide obtained from 6 mg of ferriporphyrin c-peptide was dissolved in 0.5 ml water and adjusted to a pH of 7 to 8 by dropwise addition of 0.05 *M* ammonia. A solution of 0.2 mg subtilisin in 0.1 ml water was added and the mixture incubated at 37° for 23 hrs. After heating for 3 minutes in a boiling water-bath to inactivate the enzyme, the solution was evaporated to dryness *in vacuo*. No preliminary fractionation was necessary and the digest was investigated directly on paper chromatograms.

The peptic split products of salmon and chicken cytochromes. Salmon and *chicken* cytochromes c were digested with pepsin in the same way as has been described above for beef cytochrome. In the purification involving partition chromatography on Hyflo Supercel, the ferriporphyrin c-peptides obtained did not differ in their behaviour from the cor-

Table 2. Iron and nitrogen determinations.

Ferriporphyrin c-peptide preparation	% Fe		% N		Mol.wt		Molar ratio N : Fe	
	found	calcd.	found	calcd.	found (based on Fe content)	calcd.	found	calcd.
Beef prepn. 1	2.70	2.97 *			2 068	1 879 *		
Beef prepn. 2	2.85	2.97 *			1 959	1 879 *		
Beef prepn. 3	2.76	2.97 *	13.85	14.91 *	2 023	1 879 *	19.99	20
Salmon	2.76	2.97 *			2 023	1 879 *		
Chicken	2.77	2.95**			2 016	1 895**		

* Based on the formula $C_{50}H_{84}O_{17}N_{16}S_2 \cdot C_{34}H_{32}O_4N_4Fe+OH^-$.
** Based on the formula $C_{50}H_{84}O_{18}N_{16}S_2 \cdot C_{34}H_{32}O_4N_4Fe+OH^-$.

responding peptide of bovine origin. The amino acid composition and the iron contents of the three haemopeptides are compared in Tables 1 and 2. After splitting with silver salt in acetic acid, the porphyrin-free peptides were subjected to further digestion with subtilisin and the resulting mixtures of lower peptides investigated by paper chromatography.

RESULTS AND DISCUSSION

In the hydrolysate of the purified ferriporphyrin c-peptide of *bovine* origin, 6 amino acids were identified by paper chromatography: glutamic acid, threonine, alanine, histidine, valine and lysine (Table 1). For comparison, it may be noted that the peptic degradation product of cytochrome c as obtained by the isolation procedure of Tsou [1], which did not include a partition chromatographic purification, contained aspartic acid, glycine, leucine, phenylalanine, proline and serine [2,3] in addition to the six amino acids found in the purified preparation.

A quantitative analysis by the method of Moore and Stein [17] gave evidence that the peptide was composed of one residue of threonine, alanine, histidine and lysine, while valine occurred twice and glutamic acid three times (Table 1). Neither paper chromatography nor ion exchange chromatography revealed the presence of cysteine or cystine, but this is not inexplicable since it is known that the two sulphur containing amino acid residues linked to the prosthetic group of cytochrome c are not set free by mild acid hydrolysis, but remain combined with the pigment in the form of porphyrin c [31,9]. However, after fission of the thioether bridges by the silver salt method of Paul [25] and oxidation of the —SH groups to —SO_3H groups, the porphyrin-free peptide moiety, after hydrolysis with acid, yielded cysteic acid in addition to the other six amino acids. Since the cysteic acid found is derived from two porphyrin-bound cysteine residues, and since the six other amino acids account for nine amino acid residues, it can be concluded that the peptide component of the peptic degradation product of cytochrome c is composed of 11 amino acid residues.

In the ferriporphyrin c-peptide only one α-amino group was found free to react with fluorodinitrobenzene; after dinitrophenylation and hydrolysis, one of the two valine residues was recovered in the form of DNP-valine. After short hydrolysis, however, DNP-Val-Glu was found in high yield. This indicates that the 11 amino acid residues of the ferriporphyrin-c-peptide are arranged in one single peptide chain having the dipeptide sequence Val-Glu in the N-terminal position. In contrast, Tsou [2] had found that his "pepsin-modified cytochrome c" contained 3.4 α-amino nitrogen atoms per atom of iron.

On treatment of the ferriporphyrin c-peptide with trypsin, a small fragment was split off which, on acid hydrolysis, yielded valine, glutamic acid and lysine. DNP treatment showed valine to be the N-terminal residue. Lysine, on the other hand, could be assigned the C-terminal position since trypsin is known to split specifically peptide bonds involving the carboxyl groups of lysine and arginine residues [32-34,4]. A tripeptide containing one glutamic acid and one lysine residue would be expected to behave as a neutral substance at a pH of 6 to 7; the peptide under investigation, however, at a pH of 6.4, proved to be a basic substance. This suggests that the glutamic acid residue is not present as such, but is in the form of its amide, glutamine, and that the tripeptide is Val—Glu(NH_2)—Lys.

Table 3. Partial acid hydrolysis of bovine "porphyrin-free peptide". Peptides from the acidic fraction (see Fig. 1.).

Spot No. (Fig. 1)	Colour with ninhydrin	Amino acids present after hydrolysis	After DNP treatment and hydrolysis		Structure *
			DNP amino acids	amino acids	
1 **	purple	$CySO_3H$ (× × × ×)			cysteic acid
2 **	purple	$CySO_3H$ (× × ×) Glu (× × ×)	DNP-Glu (× ×)	$CySO_3H$ (×)	Glu-$CySO_3H$
3	purple	$CySO_3H$ (×) Glu (×) His (×)	DNP-Glu (×) im-DNP-His (×)	$CySO_3H$ (×)	Glu-[$CySO_3H$, His]
4	yellow → purple	$CySO_3H$ (×) Glu (× ×) Ala (×)			[$CySO_3H$, Ala, Glu]
5	yellow → purple	$CySO_3H$ (× ×) Ala (× × ×)	DNP-$CySO_3H$(× ×)	Ala (× ×)	$CySO_3H$-Ala
6	purple	Glu (× × ×)			glutamic acid
7	purple	Glu (×) Ala (×)	DNP-Ala (?)	Glu (?)	Ala-Glu
8	purple	Glu (×) Val (×)	DNP-Val (?)	Glu (?)	Val-Glu

* Square brackets indicate that the sequence of amino acids inside the brackets has not been established.

** Spots 1 and 2 can be separated by prolonged chromatography in butanol-acetic acid.

Further information about the amino acid sequence in the ferriporphyrin c-peptide was obtained by hydrolysis of its porphyrin-free peptide moiety and investigation of the split products. The results obtained with the acidic and neutral fractions of a partial acid hydrolysate are recorded in Figs. 1 and 2 and Tables 3 and 4. Fig. 3 and Table 5 give results obtained through the use of the proteolytic enzyme subtilisin.

Special attention must be given to peptides 3 and 4 in Fig. 3 and Table 5. These two dipeptides would seem to be identical with the dipeptides 7 and 8 (Ala—Glu and Val—Glu) found in the acid hydrolysate (Fig. 1 and Table 3), but they differ markedly in their R_F values. Whereas the peptides 7 and 8 (Fig. 1) show chromatographic properties previously found to be exhibited by Ala—Glu [4] and Val—Glu [29], the dipeptides 3 and 4 of the enzymic digest (Fig. 3) are travelling faster in phenol and slower in butanol-acetic acid. This behaviour appears to be characteristic of peptides containing glutamine rather than glutamic acid, and it may be mentioned that free glutamine shows the same difference in chromatographic properties with respect to free glutamic acid. The conclusion that peptides 3 and 4 of the enzymic digest are Ala—Glu(NH_2) and Val—Glu(NH_2) is strongly supported by the known

Table 4. Partial acid hydrolysis of bovine "porphyrin-free peptide". Peptides from the neutral fraction (see Fig. 2).

Spot No. (Fig. 2)	Colour with ninhydrin	Amino acids present after hydrolysis	After DNP treatment and hydrolysis		Structure
			DNP amino acids	amino acids	
1 *	purple	$CySO_3H$ (× ×) Lys (× × ×)	di-DNP-Lys (×)	$CySO_3H$ (× ×)	Lys-$CySO_3H$
2 *	yellow → grey	$CySO_3H$ (× × ×) His (× × × ×)	DNP—$CySO_3H$ (× × ×) im-DNP-His (× × × ×)		$CySO_3H$—His
3	purple	Glu (×) Ala (×)	DNP-Ala (×)	Glu (×)	Ala-Glu
4	purple	Ala (× ×)			alanine
5	purple	Glu (× ×) Val (× ×) Lys (× ×)	DNP-Val (× ×) ε-DNP-Lys (× ×)	Glu (× ×)	Val-[Glu, Lys]
6	purple	Glu (× ×) Val (× ×)	DNP-Val (×)	Glu (×)	Val-Glu
7	grey-brown	Glu (× × ×) Thr (× ×) Val (× × ×)	DNP-Thr (×)	Glu (× ×) Val (×)	Thr-[Val, Glu]

* Spots 1 and 2 were separated well when collidine-lutidine was used as the second solvent instead of butanol-acetic acid.

proteolytic specificity of subtilisin; this bacterial enzyme has previously been reported to attack peptide bonds involving the carboxyl groups of glutamine and asparagine residues [35, 36], whereas the amide groups are left intact. Mineral acid, on the contrary, readily breaks amide bonds.

In Table 6 all the peptides are listed which have been obtained from the peptic degradation product of bovine cytochrome c. From their structure the following sequence of amino acids can be deduced:

$$Val—Glu(NH_2)—Lys—CySO_3H—Ala—Glu(NH_2)—CySO_3H—His—Thr—[Val\ Glu].$$

In this sequence the order of the last two residues has not yet been established in a straightforward manner. It can be assumed, however, that the order is not different from that occurring in the corresponding porphyrin-free peptide derived from the *tryptic* degradation product of cytochrome c [4, 10],

$$CySO_3H—Ala—Glu(NH_2)—CySO_3H—His—Thr—Val—Glu—Lys,$$

since both peptides have their origin in the same part of the protein moiety of cytochrome c. The perfect agreement between the two enzymic degradation products as regards amino acid sequence is clear evidence that no rearrange-

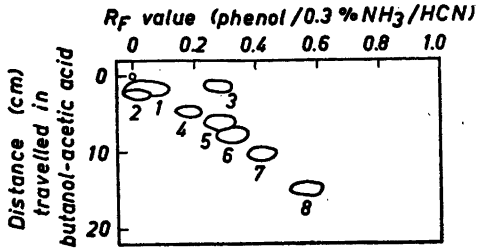

Fig. 1. Partial acid hydrolysate of bovine "porphyrin-free peptide". Chromatogram of the acidic fraction (see Table 3).

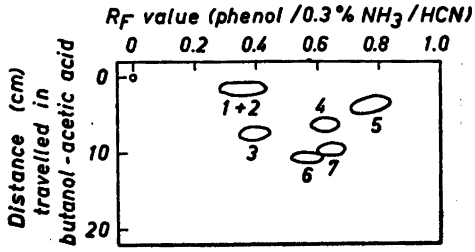

Fig. 2. Partial acid hydrolysate of bovine "porphyrin-free peptide". Chromatogram of the neutral fraction (see Table 4).

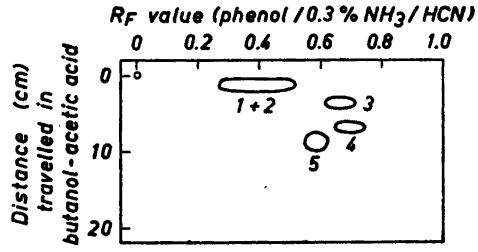

Fig. 3. Chromatogram of subtilisin hydrolysate of bovine "porphyrin-free peptide" (see Table 5).

ment has taken place within the peptide chain during enzymic digestion. Taking this for granted and remembering that the two cysteic acid residues of the porphyrin-free peptide are derived from two porphyrin-bound cysteine residues, the sequence of amino acids in the peptic degradation product of bovine cytochrome is concluded to be

$$\text{Val—Glu}(NH_2)\text{—Lys—CySH—Ala—Glu}(NH_2)\text{—}$$
$$\text{CySH—His—Thr—Val—Glu.}$$

On the basis of this peptide formula and of the known nature of the porphyrin component of cytochrome c[7], it is possible to calculate the iron and nitrogen contents of the peptic degradation product. In Table 2 the calculated figures are compared with the values actually found by analysis. The latter values prove to be considerably lower than expected although the iron content (2.76 %) is obviously far superior to that reported by Tsou (2.21 %) [2]. However, the molar ratio between the values found for nitrogen and iron is in excellent agreement with the theoretical figure of 20. This gives definite indication

Table 5. Subtilisin hydrolysis of bovine "porphyrin-free peptide" (see Fig. 3).

Spot No. (Fig. 3)	Colour with ninhydrin	Amino acids present after hydrolysis	After DNP treatment and hydrolysis		Structure
			DNP amino acids	amino acids	
1 *	purple	$CySO_3H$ (× × ×) Lys (× × ×)			[Lys, $CySO_3H$]
2 *	yellow → grey	$CySO_3H$ (× ×) His (× ×)			[$CySO_3H$, His]
3	purple	Glu (× × ×) Ala (× ×)	DNP-Ala (× ×)	Glu (× × ×)	Ala-Glu
4	purple	Glu (× × ×) Val (× × ×)	DNP-Val (× × ×)	Glu (× × ×)	Val-Glu
5	brown-yellow	Glu (× × ×) Thr (× ×) Val (× × ×)	DNP-Thr (× × ×)	Glu (× × ×) Val (× × ×)	Thr-[Val, Glu]

* Spots 1 and 2 were eluted from the 2-dimensional chromatogram (Fig. 3) and separated by 1-dimensional chromatography using collidine-lutidine as the solvent.

that the haemopeptide preparation is free from contaminating nitrogenous substances such as peptide impurities, though not free from other substances which may increase the molecular weight and lower iron and nitrogen values, such, for instance, as inorganic or organic (*e.g.*, phthalate) ions.

In Tables 1 and 2 results are included which have been obtained with the peptic degradation products of *salmon* and *chicken* cytochromes. It can be seen

Table 6. Peptides obtained from the peptic degradation product of bovine cytochrome c.

DNP-technique	DNP-Val-Glu
Digestion with trypsin	Val-Glu(NH_2)-Lys
Hydrolysis with HCl	Val-Glu $CySO_3H$-Ala $CySO_3H$-His Val-[Glu, Lys] Ala-Glu Thr-[Val, Glu] Lys-$CySO_3H$ Glu — $CySO_3H$ [$CySO_3H$, Ala, Glu] Glu — [$CySO_3H$-His]
Digestion with subtilisin	Val-Glu(NH_2) Ala, Glu(NH_2) Thr-[Val, Glu] [Lys, $CySO_3H$] [$CySO_3H$, His]
Structure of the "porphyrin-free peptide"	Val-Glu(NH_2)-Lys-$CySO_3H$-Ala-Glu(NH_2)-$CySO_3H$-His-Thr-[Val, Glu]

that the iron contents of these two ferriporphyrin c-peptide preparations were found to be virtually the same as those obtained for the bovine material. In the amino acid composition, the *salmon* haemopeptide did not differ at all from the bovine one, whereas *chicken* ferriporphyrin c-peptide did not contain alanine, and serine was found instead. In all three haemopeptide preparations, the same N-terminal dipeptide sequence, Val—Glu, was found to be present.

To get further information, the peptic degradation products of salmon and chicken cytochromes c were split with silver salt and oxidized with performic acid, using the same methods as described above for bovine preparations. The porphyrin-free peptides thus obtained were subjected to the proteolytic action of subtilisin. In the enzymic digest of the *salmon* peptide the following 5 split products were found to be present preeminently:

Val—Glu(NH_2), Lys—$CySO_3H$, Ala—Glu(NH_2), $CySO_3H$—His and Thr—[Val, Glu].

These peptides being identical with the ones identified in the subtilisin hydrolysate of the corresponding bovine peptide preparation (*cf.* Table 6), the sequence of amino acid residues in the peptic degradation product of salmon cytochrome c appears to be the same as in bovine cytochrome,

Val—Glu(NH_2)—Lys—CySH—Ala—Glu(NH_2)—CySH—His—Thr—Val—Glu.

Subtilisin treatment of the *chicken* peptide also yielded

Val—Glu(NH_2), Lys—$CySO_3H$ and Thr—[Val, Glu];

instead of Ala—Glu(NH_2), however, Ser—Glu(NH_2) was found. This is taken to indicate that the amino acid sequence in the peptic degradation product of chicken cytochrome c will be

Val – Glu(NH_2) – Lys – CySH – Ser – Glu(NH_2) – CySH – His – Thr – Val – Glu *.

The fact that an alanine residue occurring in beef and salmon cytochromes c and previously shown to be also present in horse and pig cytochromes [4], is replaced by a serine residue in a bird cytochrome, is rather surprising. It is the more interesting, since an amino acid residue with a side-chain devoid of a polar group appears to be substituted by another residue possessing a polar group in its side chain, and since the amino acid residue found to be replaceable is situated in the very neighbourhood of the prosthetic group. Certainly, this is a remarkable example of how the species specificity of protein structure may be explicable in terms of differences in amino acid sequence, and the question arises what the genetic implication of such differences may be.

* Further evidence for this sequence was adduced by subjecting the porphyrin-free peptide (obtained from the peptic degradation product of chicken cytochrome c) to partial hydrolysis with hydrochloric acid and investigating the split products present in the hydrolysate. The following peptides were found and characterised: Val-Glu, Val-[Glu,Lys], Val-[Glu, Lys, $CySO_3H$], Lys-$CySO_3H$, Ser-Glu, Ser-[Glu, $CySO_3H$], Ser-[Glu, $CySO_3H$, His], $CySO_3H$-His, Thr-[Val, Glu].

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Study of a Peptic Degradation Product of Cytochrome c

II. Investigation of the Linkage between Peptide Moiety and Prosthetic Group

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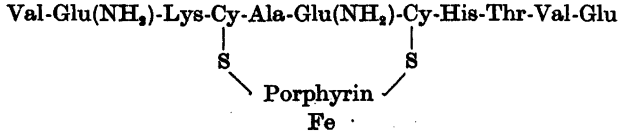
The ferriporphyrin c-peptide obtained by peptic digestion of cytochrome c has been subjected to titrimetric, spectrophotometric and magnetic measurements. The results are compared with corresponding data for cytochrome c. In contradistinction to its parent protein, ferriporphyrin c-peptide is not altered spectrophotometrically by chloride ions at low pH values. Whereas the acid compound of ferricytochrome is transformed to the neutral form by a divalent dissociation with a pK of 2.12, it was found that the corresponding transformation of the peptide takes place in two separate steps with a pK of 3.4 and 5.8, respectively. The iron of the acid form has a susceptibility of 11 900 $\times$ 10^{-6} cgs emu and hence essentially ionic bonds. The alkaline form is covalent with a susceptibility of 2 100 $\times$ 10^{-6} cgs emu. The intermediate form (present between pH 3.4 and 5.8) has a tentative susceptibility of 8 800 $\times$ 10^{-6} cgs emu, and its structure is uncertain. The experimental data are related to structural differences between the peptide and cytochrome c, and the nature of the heme-linked groups is discussed. At pH 4.9 the ferriporphyrin c-peptide exhibits a peroxidatic activity more than 20 times stronger than that of cytochrome c.

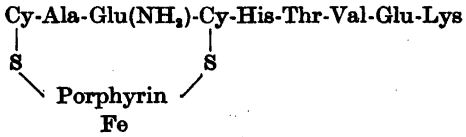
Attempts to understand the biological activity of a protein must be firmly based on considerations of the chemical structure of the molecule. Since many heme proteins have identical prosthetic groups [1], the activity associated with a particular heme protein must be a reflection of specific relationships existing between the prosthetic group and the protein moiety. Theorell and coworkers have for many years been concerned with the chemistry of heme proteins including cytochrome c. In 1939, Theorell [2] presented evidence suggesting two stable thioether bridges between the heme prosthetic group and the cytochrome c-protein. Data accumulated since that time strongly indicate the existence in fact of such bridges. In 1941, on the basis of titrimetric, spectrophotometric, and magnetic investigations, Theorell and Åkeson [3] proposed, in addition, two linkages between the iron atom of the prosthe-

tic group and imidazole groups of histidine residues in the protein moiety. Further investigations have substantiated this concept, and the nature of the iron-nitrogen linkages at various pH's has been clarified [4,5].

With the development of convenient methods for the preparation of relatively large amounts of pure cytochrome c[6] it has now become possible to study in greater detail certain structural aspects of the cytochrome c-protein itself. By controlled digestion with proteolytic enzymes [7], fragments of the protein firmly bound to the prosthetic group have been obtained. In the foregoing paper [8] the purification and structure of a peptic degradation product has been described:



Tuppy and Bodo [9] have also studied the structure of a tryptic degradation product:



The two fragments have certain important structural features in common. The porphyrin is bound *via* thioether bridges to two cysteine residues. In addition, a histidine residue is attached to one of the cysteine residues in the polypeptide sequence. The imidazole group of this histidine has been suggested by one of us to be one of the groups linked to the iron atom of the heme in the intact cytochrome c molecule [9].

This paper is concerned with an investigation of the peptic degradation product using the same physico-chemical methods which have been applied to the intact cytochrome c molecule.

MATERIAL AND METHODS

All the experiments were performed with the peptic degradation product of *cow* cytochrome c described in the foregoing paper [8]. The product is easily autoxidizable and is therefore obtained as a *ferriporphyrin* c-peptide. The iron content was determined by a modification of the method given by Lorber [10]. It was found to be 2.85 % in most of the preparations. All molarities are calculated on iron basis.

The titration apparatus described by Paléus was used [5]. N_2O or N_2 was passed through the titration vessel in order to diminish CO_2 errors. The pH of the solution was measured with a Radiometer Model 22 pH meter. The spectrophotometric experiments were carried out at 20° in a Beckman Model DU spectrophotometer. The absorption data are given as $\beta = d^{-1} \cdot c^{-1} \cdot \ln I_0/I$ cm²/mole, where natural logarithms are used, d is the optical path length in cm and c is the concentration in moles/cm³.

Especially when containing higher concentrations of salt and in certain pH regions the absorption values of the peptide solutions showed a tendency to decrease with time in an irreproducible manner. This troublesome behaviour of the peptide was eliminated to a large extent by carefully washing all vessels and absorption cuvettes with dilute ammonia and distilled water, and by rinsing them several times with the solution to be investigated.

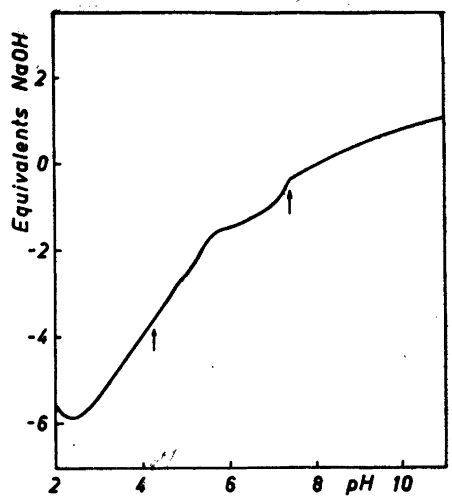


Fig. 1. Titration curve of the peptic degradation product of cow cytochrome c. The two arrows indicate the range of pH where the peptide precipitated.

The magnetic determinations were made in the apparatus constructed by Theorell and Ehrenberg[11]. A dilute solution of nickel chloride was used as a paramagnetic standard, $\chi_M = 4\,434 \times 10^{-6}$ cgs emu at 20° C, at which temperature all the measurements were made.

EXPERIMENTS AND RESULTS

Titrations

Approximately 3.5 mg of the peptide were suspended in 2.5 ml of water and dissolved by the addition of 1 *N* H_2SO_4 to obtain pH 2. The sample was then titrated with 1 *N* KOH. Under these conditions the peptide regularly precipitated at a pH of about 4.3 and did not redissolve until about pH 7.4. In this region the pH values, as measured immediately after each addition of alkali, were unstable but attained a constant value after longer waiting.

Several titrations were made with the same peptide preparation. It appeared difficult to reproduce the titration curve exactly, especially at the extreme pH values. One titration curve, corrected for blank, is shown in Fig. 1. The main shape of that curve was constantly reproduced and all the experiments indicated that about 7 equivalents were titrated between pH 2 and 11. This is in agreement with the structure of the peptide which has 7 titratable groups. Using the following tentative p*K* values: 3.1 and 4.4 for the carboxyls of glutamic acid, 4.6 for the two propionic acid residues, 6.3 for the imidazole of the histidine, 7.6 for the α-amino group of valine and 9.6 for the ε-amino group of lysine, a theoretical titration curve could be constructed which agreed nicely with the experimental observations except for the S-shaped part in the pH region 6—7.5. It has been suggested that the p*K* of the propionic acid residues in cytochrome c is about 5.5[2,5]. If this value is applied in our case, the p*K* of histidine must be shifted down to as far as 3.5, in order to obtain the same degree

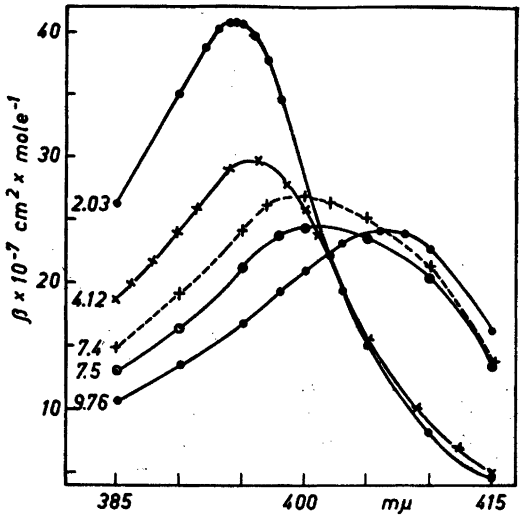


Fig. 2. Soret band of the peptide at different pH values. The pH is indicated at the beginning of every curve. The drawn curves are made from an acid stock solution, the dotted curve is from an alkaline stock solution. Cf text.

of coincidence with the experimental curve. The S-shaped part around neutrality remains unexplained, but might be a result of the precipitation and redissolution of the peptide.

Spectrophotometric investigations

The insolubility of the peptide made it difficult to obtain spectrophotometric data in the visible range of the spectrum. All the measurements were therefore made in the Soret band region.

a. The effect of the chloride ion concentration on the absorption spectrum in the Soret band region.

In order to determine the influence of anions on the absorption spectrum, aliquots of a 5 μM solution of the ferriporphyrin c-peptide in 0.001 N HCl (pH 3.03) were diluted with equal volumes of 0.001 N HCl containing varying concentrations of NaCl. In contrast to the findings of Boeri, Ehrenberg, Paul and Theorell [4] with intact cytochrome c, the position of the absorption maximum of the ferriporphyrin c-peptide at 395 mμ was not influenced by the Cl^- concentration. Furthermore, as shown in Fig. 4, the extinction at this wave length was not influenced by Cl^- concentrations between 0.001 and 0.3 M. At higher Cl^--concentrations a moderate decrease in absorption was observed. The same results were obtained in experiments at pH 2.00.

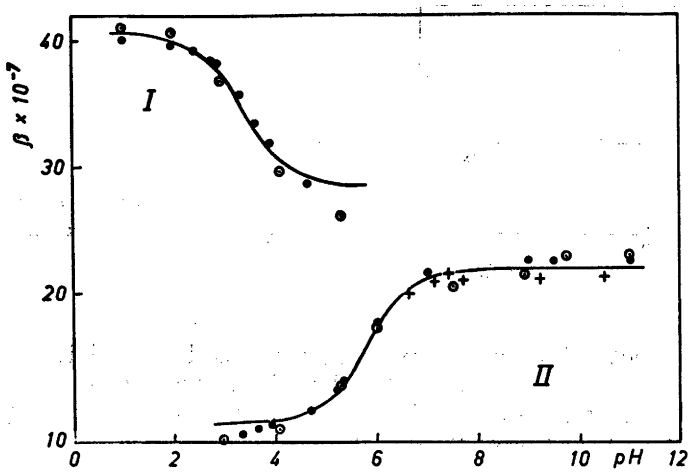


Fig. 3. Extinctions at 395 mμ (curve I) and 410 mμ (curve II) plotted versus pH. The different points are from different sets of experiments.

b. The effect of hydrogen ion concentration on the absorption spectrum in the Soret band region.

A 2.5 μM solution of peptide (2.85 % Fe) in 0.01 M HCl was prepared. To aliquots of this stock solution, either 3 N HCl or 1 N KOH was added from an "Agla" micrometer syringe, until the desired pH was obtained. Fig. 2 shows that in the strongly acid range the Soret band has a high maximum at 395 mμ. As the pH was increased towards 5.0, the extinction at 395 mμ decreased conspicuously, while the absorption maximum shifted only slightly towards the visible region. As the pH was increased above pH 5, however, the absorption maximum shifted markedly towards longer wavelengths, whereas the extinction changed only slightly.

Another set of curves were measured starting with a stock solution, 6.51 μM peptide (new preparation: 2.76 % iron) and 10^{-4} N NaOH. Aliquots of this stock solution were mixed with the double volume of acid, water or mixtures of water and NaOH to give different pH-values. At the most acid or alkaline pH's the new curves are almost identical with the corresponding ones of Fig. 2. One curve with an intermediate pH is included in that figure. It clearly shows the difficulty to obtain coinciding curves in this pH region where, however, the measured pH values of these unbuffered solutions are very unreliable.

In Fig. 3 the absorption values at 395 mμ and 410 mμ are plotted *versus* pH. The data at 395 mμ between pH 1 and 4.5 fit satisfactorily with a normal dissociation curve I with $n = 1$, $pK = 3.4$ and the asymptotic levels of $\beta_{395} = 40.5 \times 10^7$ cm²/mole at low pH and 28.5×10^7 cm²/mole at high pH. Also the data at 410 mμ are in fair agreement with a normal dissociation curve II: $n = 1$, $pK = 5.8$, $\beta_{410} = 9.5 \times 10^7$ cm²/mole and 21.9×10^7 cm²/mole at low and high pH, respectively.

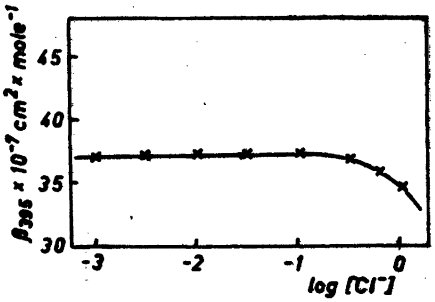


Fig. 4. The extinction at 395 mμ as a function of [Cl⁻] at pH 3.0.

From the spectrophotometric experiments just described it is clear that three forms of the polypeptide can be identified, one acid, one alkaline and one intermediate form. Because of the proximity between the two pK values the latter form is never present to 100 %.

Magnetic experiments

The following experiments were undertaken to determine the paramagnetic susceptibility of the three forms of the polypeptide described above.

About 2 mg of the dry polypeptide were accurately weighed in a small glass beaker, 1 ml of water was added and the sample was acidified by slow addition of 3 *N* HCl from a micrometer syringe, during rapid stirring by means of a magnetic stirrer. The pH was measured with a carefully cleaned and dried glass electrode and adjusted to about 1.5. The sample was immediately transferred from the open beaker to a closed tube. A large amount of a buffer with the same proportions of water and acid was prepared and measured as blank in the magnet. After that the sample was measured and then brought back to the closed tube. The blank was read again.

Largest possible amount was accurately pipetted over to the cleaned beaker and the pH of that sample was adjusted to between 3.7 and 4.0 by 3 *N* NaOH added from a micrometer syringe. Higher pH values must be avoided because of the risk of precipitation. The sample was transferred to a closed tube, a proper blank buffer was prepared from the old buffer and the magnetic measurements made.

Table 1. Paramagnetic susceptibilites of the iron of the ferri porphyrin peptide at different pH values in the presence of chloride or sulfate ions.

HCl			H_2SO_4		
pH	c_{Cl^-} m*M*	$\chi_{Fe} \times 10^6$ cgs emu	pH	$c_{SO_4^{2-}}$ mM	$\chi_{Fe} \times 10^6$ cgs emu
1.50	37	11 360	1.50	32	11 960
3.70	37	9 600	3.90	31	9 550
10.60	37	1 930	10.90	31	2 230
1.50	68	12 750	1.40	91	11 420

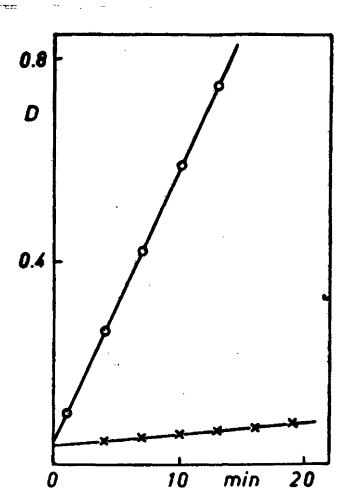


Fig. 5. The peroxidatic activity of the peptide (o) and cytochrome c (×).

A measurement was similarly made at pH 10.5—11.0 and finally the sample was again brought to pH 1.5 and remeasured. An iron determination was made on the rest of the sample.

The same procedure was repeated on a new sample but with hydrosulfuric acid instead of hydrochloric acid.

From the iron content of the dry peptide and the dilutions made, it is easy to calculate the concentration of iron in every magnetic determination. In both sets of experiments, however, the direct determination of iron in the sample after the last measurement in the magnet showed 4 % higher iron content than calculated. This is probably due to evaporation and an additive correction of 1 % per step in the treatment of the sample was introduced.

Because of the high iron content of the polypeptide the diamagnetic contribution is of comparatively small importance. A rough value for it, 100×10^{-6} cgs emu, was obtained by analogy with known figures of various heme proteins studied in this laboratory.

The susceptibilities determined are listed in Table 1. No definite influence by the anions can be seen at the low pH. According to the spectrophotometric investigation the acid form is present to practically 100 % at pH 1.5 and the alkaline form at pH above 10.5. We thus obtain $\chi_{acid} = 11\,900 \times 10^{-6}$ and $\chi_{alkaline} = 2\,100 \times 10^{-6}$ cgs emu. From these values, the data at pH 3.70 and 3.90 and the two pK values of 3.4 and 5.8, we calculate $\chi = 8\,800 \times 10^{-6}$ cgs emu as a tentative value of the susceptibility of the intermediate form.

Peroxidatic activity

The procedure designed by Paul and Avi-Dor [12] for the assay of horseradish peroxidase, in which mesidine serves as hydrogen donor, was used in a simplified form to compare the known slight peroxidatic activity of cytochrome c with that of its peptic degradation product.

Two flasks, each containing 35 ml 0.1 *M* acetate buffer pH 4.9, 3 ml of an aqueous 0.2 *M* mesidine hydrochloride solution, and 2 ml 0.1 *M* hydrogen peroxide, were cooled down to 0° in a bath containing crushed ice. Then 1.0 ml of 0.2 m*M* solutions of bovine cytochrome c (0.433 % Fe) and of the ferriporphyrin c-peptide in 0.05 *M* NH_3 were added. From the reaction mixtures kept at 0°, after different time intervals, aliquots were taken out and their extinctions at 490 mμ measured immediately in a Beckman spectrophotometer. In Fig. 5 the extinction readings are plotted *versus* time. The slope of the straight lines obtained is proportional to peroxidatic activity.

DISCUSSION

As a consequence of their investigations, Theorell and coworkers have suggested a conceptual picture of cytochrome c which reflects its observed properties. The prosthetic group can be imagined to be a hematin disc embedded in a crevice of the protein moiety. The iron in the prosthetic group is coordinated to two haemochromeforming groups in addition to the four porphyrin nitrogen atoms. Presumably because of the structural architecture, the iron atom is inaccessible to molecular oxygen and is therefore non-autoxidizable. At the same time, electron transfer is made possible.

At acid reactions, however, the covalent bonds between the iron and the hemochrome forming groups of ferri-cytochrome c are ruptured (p*K* 2.12) [4], and the bonds to the iron assume an essentially ionic character. Chloride ions can enter yielding a dichloride derivative of the acid ferri-cytochrome c which has a peculiar structure around the iron atom with covalent bonds to the four pyrrolic nitrogens and two ionic bonds perpendicular to the heme disc (Boeri, Ehrenberg, Paul and Theorell [4]). The unusual stability of this structure, and the fact that even at low pH values, the protein moiety and prosthetic group do not dissociate are presumably due to the stable thioether bridges.

This investigation reveals that there are some interesting similarities and dissimilarities between the native cytochrome c and its peptic degradation product.

The spectrum of the ferriporphyrin peptide in the Soret band region is at very low pH values quite similar to that of acid ferricytochrome c ("cyt-$2H^+$", Boeri *et al.*[4]). The absorption maximum of both compounds is situated at 395 mμ; the absorption coefficient ($\beta \times 10^{-7}$ cm^2/mole) is about 41 for the peptide whereas Boeri *et al.*, by extrapolation to $[Cl^-] = 0$, calculated it to be around 50 for "cyt - $2H^+$".

The magnetic susceptibility of the two compounds are not equal. For the peptide χ_{Fe} was measured to be 11 900 $\times 10^{-6}$ cgs emu, and the extrapolated value for "cyt - $2H^+$" was 16 200 $\times 10^{-6}$ cgs emu. Both values are, however, compatible with the presence of 5 odd electrons in the iron atom, and the bonds involved are essentially ionic. The difference in paramagnetic susceptibility might be reflected in heme-linked groups of different nature.

As shown here, contrary to the findings in the intact enzyme, the peptic degradation product of cytochrome c does not exhibit a "chloride effect", its spectrophotometric properties in the Soret band region remain unchanged on

increase of the chloride ion concentration up to a molarity of 0.3. The failure of chloride ion to be incorporated between iron and hemochrome forming groups of the peptide can be attributed to the lack of the firm architecture present in the undenatured cytochrome c molecule when the covalent bonds between them and the iron are broken.

An increase in pH leads to a simultaneous release of 2 protons from "Cyt $-2H^+$" (pK 2.12) and the formation of neutral cytochrome c. This behaviour has been interpreted as indicating that the two hemochrome-forming nitrogenous groups of the protein moiety of cytochrome c are identical, and are symmetrically attached and equally firmly bound to the iron. In the peptide investigated, only one histidine is present, and the protons are, as might be expected, not released simultaneously. The first is released at a pK of 3.4 and the second at 5.8.

No further spectral changes could be derived in the Soret band region at pH's below 11. The alkaline form of the peptide has its absorption maximum at 406 mμ with $\beta = 24.5 \times 10^{-7}$ cm^2/mole, which is nearly identical with the corresponding values of neutral cytochrome c, 407 mμ and $\beta = 26.0 \times 10^{-7}$ cm^2/mole. Also the paramagnetic susceptibilities are in close agreement, 2 080 (mean value) and 2 120 $\times 10^{-6}$ cgs emu, respectively. Our conclusion is that the iron in the alkaline form of the peptide is held by six octahedral covalent bonds. It is interesting to observe that the great similarity of paramagnetism and absorption properties in the Soret region do not correspond to identical groups coordinated to the iron. In cytochrome c two histidine residues are supposed to be bound but in the peptide only one histidine is present and could possibly be heme-linked.

The intermediate compound (appearing between pH 3.4 and 5.8) of the peptic degradation product has its Soret maximum at about 396 mμ with $\beta \sim 30 \times 10^{-7}$ cm^2/mole. The paramagnetic susceptibility of this compound is found to be about 8 800 $\times 10^{-6}$ cgs emu. In this case no close analogy could be drawn with any compound of native cytochrome c. It is true that the ferricytochrome dichloride (cyt. $- 2H^+ - 2Cl^-$) has a paramagnetism of comparable magnitude but it is somewhat lower, and its Soret band is situated at 401 mμ. Probably the most suitable assumption is nevertheless that the susceptibility 8 800 $\times 10^{-6}$ cgs emu is due to a structure with 3 odd electrons in the iron atom as in the ferricytochrome dichloride. This type of compound is rare and the types of bonds involved are but little known. The possibility of 5 odd electrons and essentially ionic bonds cannot, however, be totally excluded. In any case the transition with pK = 3.4 means that one heme-linked group is titrated and that the bonds to the iron are more or less profoundly changed.

The present investigations do not give any unambiguous indication of the nature of the heme-linked groups of the ferriporphyrin c-peptide. The structure of the peptide, however, limits the number of groups which can be involved. One of us (Tuppy [9]) has suggested that the histidine residue is heme-linked. From the titration curve it cannot be decided whether this group has a pK around 3.5 or 6.3. A heme-linked histidine group would be expected to have a pK lower than the "normal" value (5.5—8.0) but higher than that of ferricytochrome c (pK = 2.12) [4]. The pK of 3.4 is within that range. On the other hand it has been proposed that the imino groups of the two heme-linked

imidazoles in cytochrome c are titrated with pK's of about 9.5 [3]. The titration curve of the peptide shows that the imino group in this case cannot be titrated below pH 11. This means that either the imidazole group is heme-linked and the pK of its imino group is above 11 or the imidazole group is not heme-linked. The former possibility is in reasonable agreement with the experimental data if it is assumed that the amino group of either lysine or valine is also heme-linked with the pK shifted to 5.8. Such a shift would not distort the theoretical titration curve too much.

It is *a priori* also possible that an ionically bound carboxyl group is titrated with a pK of 3.4. The pK of 5.8 would then correspond to the titration of the imidazole or one of the amino groups. The "acidifying" effect of the heme linkage on the imidazole might be so much smaller in the peptide compared to cytochrome c as to justify the assumption that it is titrated at the lower limit of its normal region. The carboxyl group, however, does not necessarily need to be linked to the iron in the alkaline form as it could be competitively replaced by the already titrated amino group of valine without changing the titration curve. The possibility that one of the carboxyl groups of the C-terminal glutamic acid is heme-linked in the way just discussed seems much less likely than the former assumption that the imidazole of histidine and the ε-amino group of lysine or the α-amino group of valine are the heme-linked groups involved.

The configuration around the heme of the peptide might be somewhat similar to that of peroxidase. On account of this assumption the activity experiment was undertaken. It showed that the peroxidatic activity of the peptide is more than 20 times stronger than that of cytochrome c and thus about one per cent of the activity of peroxidase itself [12].

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Investigations in the Retene Field

VI.* The Composition of the Mixture of Mononitroretenes Obtained by Nitration of Retene in the Presence of Boron Trifluoride

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In a previous work it was shown that nitration of retene and subsequently chromatographic purification of the crude nitration product gave a mixture of mononitroretenes in a yield of 83 %. In the present investigation, the composition of the mixture has been determined by reducing the mononitroretenes and separating the mixed aminoretenes so obtained. By acetylation, 9-acetamidoretene (38 %) and 10-acetamidoretene (12 %) were isolated. The remaining part of the acetylation product was chromatographed and yielded 3- (4 %), 4- (16 %), 6- (2 %), 9- (4 %), and 10-acetamidoretene (4 %). Thus the mixture of nitroretenes contained 3- (4 %), 4- (16 %), 6- (2 %), 9- (42 %), and 10-nitroretene (16 %).

A simple method for the preparation of 9-acetamidoretene has been described. It includes only three operations, namely, nitration of retene, reduction of the crude nitration product, and acetylation of the mixture of aminoretenes so obtained, and gives the pure compound in a yield of 24 %.

In a previous investigation of this series [1] it was found that retene, when treated with slightly more than the theoretically required amount of nitric acid at 80—90° in a glacial acetic acid solution containing boron trifluoride, gave, after chromatographic purification of the crude product, a mixture of mononitroretenes in a yield of *ca.* 83 % (85 % if the amount of retene recovered is taken into account). In the present work attempts were made to determine the composition of the mixture.

Karrman and Bergkvist [2] nitrated retene in a mixture of glacial acetic and propionic acids at 7—10° with excess of concentrated nitric acid and obtained mixed mononitroretenes in a yield of *ca.* 60 %. By chromatography (Al_2O_3, light petroleum-benzene) they were able to separate 3-nitroretene (*ca.* 30 % of the mixture), and a mononitroretene of unknown structure ** (*ca.* 0.5 %), but

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** Proved to be 8-nitroretene. K. J. Karrman *Private communication.*

not the other components of the mixture. However, by fractional crystallization carried out in light petroleum, the process being followed using a microscope, they succeeded in separating this part of the mixture into 4-nitroretene (*ca.* 20 %) and 9-nitroretene (*ca.* 50 %).

In view of these results, it was to be expected that the mixture of mononitroretenes obtained in the boron trifluoride nitration should also contain 3-, 4- and 9-nitroretene. Before the mixture was more closely investigated, some experiments were carried out in order to find a more convenient method for the separation of the two last mentioned isomers. These experiments showed that they could indeed be separated by chromatography (Al_2O_3, light petroleum) if a big column was used. However, the mixture of mononitroretenes obtained in the nitration could not be successfully chromatographed under these conditions. Only 9-nitroretene could be separated in a pure state (*ca.* 27 %); 3- and 4-nitroretene were also obtained but were contaminated with considerable amounts of isomers. As 3-, 4- and 9-nitroretene can be separated under these conditions, the above result indicates the presence of compounds other than these three isomers.

Apparently, it is rather difficult to achieve a clear separation of the mixed mononitroretenes obtained in the boron trifluoride nitration. Attempts were made, therefore, to determine the composition of the mixture by reducing the mononitroretenes and separating the corresponding amines. By catalytic reduction with Raney nickel, 4- and 9-nitroretene can easily be converted to aminoretenes in almost quantitative yields, and this method could also be successfully used for the reduction of the mixture of mononitroretenes. The theoretically required amount of hydrogen was taken up and the mixed aminoretenes were obtained as a straw-coloured, viscous product which gradually crystallized.

Two of the amines could be separated as their N-acetyl-derivatives by taking advantage of their different rates of acetylation. Acetic anhydride was added to an ether solution of the mixed aminoretenes and in about half an hour 9-acetamidoretene (*ca.* 38 %) had separated. It was removed, and after fifteen hours another acetamidoretene (*ca.* 12 %), hitherto unknown, could be collected. When oxidized with chromic acid it yielded retenequinone and its structure must, therefore, be 10-acetamidoretene.

The rest of the acetylation product did not separate from the solution. After removal of the solvents it was obtained as a light-brown material which was found to consist chiefly of acetamidoretenes. These were successfully chromatographed on alumina using a mixture of light petroleum and ethylacetate. For a satisfactory separation alumina with relatively weak adsorptive power must be used. It should be pointed out that the acetamidoretenes show blue fluorescence and most zones could be located, therefore, by inspection of the column in ultraviolet light (glass tube with a thickness of 1 mm was used). The result of the chromatographic investigation is displayed in Table 2 (p. 380). As can be seen, fractions of relatively pure 3-, 4-, 9- and 10-acetamidoretene were obtained. Fractions 24—26 were found to be a mixture of 3- and 6-acetamidoretene which, however, could be separated on alumina using a mixture of benzene and ethylacetate — the result appears in Table 3 (p. 381). In connexion with these separations, it was observed that an inversion of the

Table 1. Compounds isolated by the acetylation of the mixture of aminoretenes and by the chromatographic separation of the products soluble in the acetylation solution.

Compound	Yield %*		
	A. Precipitated by acetylation of the mixture of aminoretenes	B. Isolated by chromatography from the soluble part of the acetylation product (*cf.* Table 2—3)	Total (A + B)
3- Acetamidoretene	—	3.7	4
4- »	—	16.6	16
6- »	—	1.7	2
9- »	38	4.0	42
10- »	12	4.5	16
Mixtures	—	16.8	17
Material lost during the operations	—	3.2	3

* Calculated on the mixture of aminoretenes.

chromatographic sequence on alumina for 3- and 9-acetamidoretene can be effected by changing the developer. Separations have been carried out with ethylacetate (8—16 % by volume) in light petroleum and with ethylacetate (0—6 %) in benzene. In the former cases 3-acetamidoretene was located above the 9-isomer while in the latter cases the inverse order was obtained.

The result of the investigation is summarized in Table 1. The yields of the acetamidoretenes obtained and thus also the composition of the mixture of mononitroretenes appear from the last column of the Table. The yields are, of course, rather approximate. This can already be seen from the fact that some of the fractions, from which the yields are calculated, were not quite pure whilst others consisted of mixtures of the separated compounds. As appears from the Table, the nitration of retene gave a mixture of mononitroretenes containing 9-, 10-, 4-, 3- and 6-nitroretene, approximately in the ratio 20 : 8 : 8 : 2 : 1. The nitration carried out by Karrman and Bergkvist [2] yielded 9-, 4- and 3-nitroretene in the ratio 20 : 12 : 8. As in the case of phenanthrene which gives 9-, 4-, 2- and 3-nitrophenanthrene [3-4] (ratio 20 : 11 : 11 : 7), 9 and 4 are the most reactive positions. From the nitration of phenanthrene, and from the theoretical calculations by Buu-Hoi, Royer, Daudel and Martin [5] according to which 2 is the most reactive position, it was to be expected that 2-nitroretene should also be formed. However, this isomer has not been detected in the nitration products of retene.

The 10-acetamidoretene isolated above was not more closely investigated but the following observations of interest may be mentioned:

1. In the acetylation experiment 10-acetamidoretene was formed much more slowly than the 9-isomer.

2. When refluxed in a mixture of ethanol and concentrated hydrochloric acid, 10-acetamidoretene was only slowly attacked and gave 10-ethoxyretene instead of the expected amine. For comparison, when treated in the same way,

the 9-isomer was hydrolysed to 9-aminoretene and 9-retenol was converted to its ethyl ether (similar etherifications are reported in the literature [6]).

These observations indicate an influence of the methyl-group of retene on a substituent in the 10-position, similar to that found by Fieser and Young [7] when retenequinone was condensed with *p*-nitrophenylhydrazine or reduced with zinc dust and acetic acid. In the former case, a reaction at the ketonic group closest to the methyl-group is retarded and only 10-hydroxy-9-(*p*-nitrophenylazo)retene is formed. In the latter case, the retenehydroquinone first formed easily splits off the hydroxy-group in the 10-position to give 9-retenol. From the fact that 1-methylphenanthrenequinone reacts in the same way, but phenanthrenequinone is reduced only to the hydroquinone under similar conditions, they concluded that it is the methyl-group which activates the bond holding the hydroxy-group.

As seen in the foregoing, 90 % of the 9-acetamidoretene separated on the acetylation of the mixed aminoretenes—the yield corresponds to 32 % calculated on retene. This method for the preparation of 9-acetamidoretene may be preferred to those reported in the literature [8,9] which include more operations and give lower yields. The mixture of mononitroretenes used as starting material in the catalytic reduction was isolated from the crude nitration product by chromatography (*cf.* Part IV [1]), this being a disadvantage of the method especially if large amounts of the 9-derivative should be prepared. The method can, however, be simplified by excluding the chromatographic purification. Certainly, a lower yield (24 % calculated on retene) is then obtained, but on the other hand only three operations are required, namely nitration of retene in the presence of boron trifluoride, catalytic reduction of the crude nitration product with Raney nickel, and acetylation with acetic anhydride of the reduction product dissolved in ether.

EXPERIMENTAL *

Separation of 4- and 9-nitroretene. A solution of 4- and 9- nitroretene (45 mg of each) in light petroleum (b.p. 40—60°) was introduced in a column of alumina (10 cm^2 × 30 cm). On passing light petroleum through the column, two faintly yellow zones were developed and successively eluted. Three fractions were collected, the first containing 9-nitroretene (38 mg, m.p. 126—27°), the second a mixture of 4- and 9-nitroretene (15 mg, m.p. 118—23°), and the third 4-nitroretene (31 mg, m.p. 135—36°).

Attempt to separate the mixed mononitroretenes obtained by the nitration of retene. The mixture (160 mg) was chromatographed on a column of alumina (10 cm^2 × 31 cm). The column was first washed with light petroleum (3.2 l) and then fifteen fractions, accounting for *ca.* 90 % of the amount adsorbed, were collected. Light petroleum was used for the elution of the first thirteen fractions but fractions 14—15 were eluted with a mixture of light petroleum (9 vol.) and benzene (1 vol.). Fractions 5—9 contained 9-nitroretene (44 mg, m.p. 122—24° to 126—27°) but all the other fractions were mixtures melting over wide ranges. However, after recrystallizations from ethanol and from

* All melting points are corrected.

light petroleum, fraction 12 (12 mg, m.p. 105—15°) gave 4-nitroretene, m.p. 136—37°, and fraction 14 (13 mg, m.p. 110—25°) 3-nitroretene, m.p. 152—53°. The isolated nitroretenes were identified by mixed melting point determinations.

4-Aminoretene was prepared by catalytic reduction of 4-nitroretene (0.28 g) in absolute ethanol (50 ml) over Raney nickel. The theoretically required amount of hydrogen was taken up, and, from the reaction solution, 4-aminoretene (0.21 g, m.p. 79—82°) was obtained. After one recrystallization from ligroin (b.p. 60—90°) it was obtained as colourless needles, m.p. 88—89°. (Found: C 87.1; H 7.59. Calc. for $C_{18}H_{19}N$ (249.3): C 86.9; H 7.69).

4-Acetamidoretene separated in a pure state when acetic anhydride was added to an ethereal solution of 4-aminoretene. It was collected after 4 hours and obtained as colourless flat needles, m.p. 201—02°. (Found: C 82.0; H 7.35. Calc. for $C_{20}H_{21}ON$ (291.4): C 82.4; H 7.26).

9-Aminoretene was prepared by reduction of 9-nitroretene (0.28 g) as described above for the 4-isomer. The crude product (0.22 g) melted at 124—25° undepressed on admixture with an authentic sample.

Reduction of the mixture of mononitroretenes. The mixture (5.6 g) was dissolved in absolute ethanol (250 ml) and reduced over Raney nickel (*ca.* 4.5 g). In 5 hours 1 370 ml (N.T.P.) of hydrogen were taken up. From the reaction solution the mixed aminoretenes were obtained as a straw-coloured viscous product (4.9 g, 99 %) which gradually crystallized. (Found: C 86.9; H 7.72. Calc. for $C_{18}H_{19}N$ (249.3): C 86.9; H 7.69).

Acetylation of the mixture of aminoretenes. The mixture (3.20 g) was dissolved in ether (100 ml) and the solution mixed with acetic anhydride (6.5 ml). Within 5 minutes crystals began to separate. After 35 minutes these were collected and washed with ether (yield: 1.42 g, 38 %, m.p. 205—06.5°). The product was recrystallized twice from toluene and melted then at 210—11°, undepressed on admixture with 9-acetamidoretene. After 15 hours another acetamidoretene which gradually separated from the acetylation solution was collected and washed with ether (yield: 0.45 g, 12 %, m.p. 216—17°). It was recrystallized from benzene and from *n*-propanol and obtained as colourless needles, m.p. 219—20°. (Found: C 82.6; H 7.42; N 4.72. Calc. for $C_{20}H_{21}ON$ (291.4): C 82.4; H 7.26; N 4.81). Its oxidation to retenequinone indicates its structure to be 10-acetamidoretene (see below).

The filtrate from the acetylation was shaken with a dilute sodium carbonate solution to remove acetic acid and acetic anhydride, washed with water and dried over sodium sulphate. After removal of the ether a light-brown friable product (1.86 g) was obtained. (Found: C 82.0; H 7.32. Calc. for acetamidoretene $C_{20}H_{21}ON$ (291.4): C 82.4; H 7.26).

In another experiment, a product (0.50 g), prepared by catalytic reduction of the crude material obtained by the nitration of retene, was dissolved in ether (10 ml) and treated with acetic anhydride (2 ml). After 1 hour the 9-acetamidoretene which had separated (0.15 g, *ca.* 24 % calculated on retene, m.p. 210—11°) could be collected. No other pure compounds separated from the reaction solution.

Separation of acetamidoretenes from the product obtained from the filtrate in the acetylation. The product (780 mg) was dissolved in a mixture of light petroleum (20 ml) and ethylacetate (10 ml) and the solution was introduced in

Table 2. Fractions collected by the chromatographic separation of the compounds which remained dissolved in the acetylation solution. Amount adsorbed = 780 mg.

Fraction No.	Mg	M. p. °C	Compound	M.p. of the pure compound °C	Yield % *
1—18	93.1	not cryst.	Mixtures	—	6.0
19—20	49.8	211—12	9-Acetamidoretene[8-9]	212—13	4.0
21	13.5	208—09			
22	8.8	165—80	Mixtures	—	1.2
23	10.3	partly cryst.			
24	18.6	165—85	Mixtures of 3- and		
25	27.2	184—92	6-acetamidoretene	—	4.4
26	23.2	200—18	(*cf.* Table 3)		
27	20.3	241—42	3-Acetamidoretene[10]	241—42	2.3
28	16.1	239—40			
29	10.7	204—17			
30	7.4	165—95	Mixtures	—	1.7
31	8.8	partly cryst.			
32	31.1	186—89			
33—35	175.6	199—200	4-Acetamidoretene	201—02	16.6
36	30.0	194—95			
37	21.5	182—86			
38	15.9	162—71	Mixtures	—	2.0
39	15.0	148—64			
40	21.2	210—13			
41	23.6	215—17	10-Acetamidoretene	219—20	4.5
42	25.9	204—08			
43	15.0	163—75	Crystalline prod.		
44—54	56.3	—	melting over	—	5.0
55—65	7.3	—	wide ranges		
1—65	746.2				

* Calculated on the mixture of aminoretenes.

a column of alumina (12 cm^2 × 30 cm). The alumina used was the commercial "Aluminium oxide, standardized for chromatographic adsorbtion analysis according to Brockmann, Merck" which had been deactivated by the addition of water (2 g/100 g of alumina). A mixture of light petroleum (7 vol.) and ethylacetate (1 vol.) was passed through the column and the filtrate collected in 150-ml portions. The results is given in Table 2. Some of the fractions from which the yields of the acetamidoretenes were calculated were somewhat impure but could be purified by recrystallizations from either toluene or

Table 3. Chromatographic separation of 3- and 6-acetamidoretene (Fractions 24—26, Table 2). Amount adsorbed = 60 mg.

Fraction No.	Mg	M.p. °C	Compound	M.p. of the pure compound °C	Yield % *
1—9	1.4	—	—	—	0.1
10	2.5	238—39			
11—12	10.2	241—42	3-Acetamidoretene	241—42	1.4
13	3.7	239—40			
14	2.3	233—35			
15	1.8	220—26			
16	1.4	203—10	Mixtures	—	0.3
17	1.5	174—85			
18	3.9	217—19			
19—22	17.0	221—22	6-Acetamidoretene [11]	225—26	1.7
23	2.2	214—16			
24	1.8	206—10			
25	1.4	198—205	Mixtures	—	0.5
26—30	3.2	—			
1—30	54.3				

* Calculated on the mixture of aminoretenes.

ethanol. The acetamidoretenes were identified by mixed melting point determinations with authentic samples.

Most (60 mg) of the material from fractions 24—26 was rechromatographed on alumina (the above mentioned commercial product). The column was washed with a mixture of benzene (19 vol.) and ethylacetate (1 vol.) and 100-ml fractions were collected. The result is given in Table 3.

Oxidation of the acetamidoretene (m.p. 219—20°) isolated from the acetylation. The compound (0.15 g) was oxidized with CrO_3 (0.35 g) in hot glacial acetic acid (3 ml). After cooling to room temperature the crystals which separated were collected, washed with 50 % acetic acid and recrystallized from glacial acetic acid giving retenequinone, m.p. 196—97°, undepressed on admixture with an authentic sample.

10-Ethoxyretene was obtained when 10-acetamidoretene (0.25 g) was refluxed for 9 hours in a mixture of ethanol (15 ml) and conc. hydrochloric acid (4 ml). On cooling colourless needles separated (0.18 g, m.p. 100—01°). These were collected and recrystallized from ethanol to give needles melting at 101—02°. (Found: C 86.3; H 8.03; C_2H_5O 16.0. Calc. for $C_{20}H_{22}O$ (278.4): C 86.4; H 7.96; C_2H_5O 16.2). When oxidized with chromic acid, as described above for 10-acetamidoretene, it yielded retenequinone.

A hydrolysis of 9-acetamidoretene was carried out under the above conditions. After 3 hours the reaction solution was cooled, diluted with water and neutraliz-

ed. The product which separated was collected, washed with water and dried. It melted at 122—24°, undepressed on admixture with 9-aminoretene.

9-Ethoxyretene. 9-Retenol (0.10 g) was refluxed for 5 hours in a mixture of ethanol (8 ml) and conc. hydrochloric acid (2 ml). The reaction solution was worked up as described for 10-ethoxyretene. The crude product (0.11 g) melted at 95—97° and the colourless needles obtained from ethanol at 98—99°. On admixture with 10-ethoxyretene it melted at 88—90°. (Found: C 86.4; H 8.00. Calc. for $C_{20}H_{22}O$ (278.4): C 86.4; H 7.96).

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Enzymatic Hydrolysis of Organophosphorus Compounds

VI. Effect of Metallic Ions on the Phosphorylphosphatases of Human Serum and Swine Kidney

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The effects of metallic ions on the phosphorylphosphatases of purified preparations of human serum and swine kidney were studied with tabun as substrate.

The serum enzyme is activated by Sr^{2+} and Ba^{2+}, the kidney enzyme by Mn^{2+} and Co^{2+}. Both types of phosphorylphosphatases are strongly inhibited by the following ions: Ni^{+2}, Pd^{+2}, Cu^{+2}, Ag^{+}, Au^{3+}, Zn^{2+} and Hg^{2+}; the serum enzyme is also inhibited by Mn^{2+} and Co^{2+}. The most potent inhibiting ion of the kidney enzyme is Ag^{+} (pI_{50} 5.55).

Some of the metallic ions studied also catalyse the non-enzymic hydrolysis of tabun; the most active ones in this respect are: Cu^{2+}, $Pd^{2+} > Au^{3+} > Ag^{+} > Ni^{2+} > Co^{2+} > Zn^{2+}$.

Mn^{2+} has a similar effect on the enzymic reactions when DFP is used as substrate. Similarly, Ag^{+} is a strong inhibitor of the DFP hydrolysis by the kidney enzyme. Sr^{2+}, on the other hand, inhibits this enzymic hydrolysis of DFP by the serum enzyme, in sharp contrast to the activating effect of this ion on the tabun hydrolysis.

Activation (by Mn^{2+}) and inhibition (by Ag^{+}) of kidney phosphorylphosphatase are time reactions. The interaction between the serum enzyme and metallic ions, on the other hand, is independent of time.

Activating and inhibiting ions compete with each other for active groups of the enzyme molecules.

After incubation of the phosphorylphosphatases of serum and kidney with metallic ions and subsequent dialysis the two enzymes behave differently.

The effects of metallic ions on the enzyme activities have been discussed from a more general point of view.

Recent papers by the present authors reported the existence and properties of phosphorylphosphatases [1]. In most of these studies tabun (dimethylamido-ethoxy-phosphoryl cyanide) was used as substrate. Employing DFP (di*iso*propoxy-phosphoryl fluoride) as a substrate and kidney as a source for "dialkylfluorophosphatase" (DFPase) in similar studies, Mounter *et al.*[2] recently reported the effects of various cations on the enzymic hydrolysis. In a preliminary comparative study of the phosphorylphosphatases of human

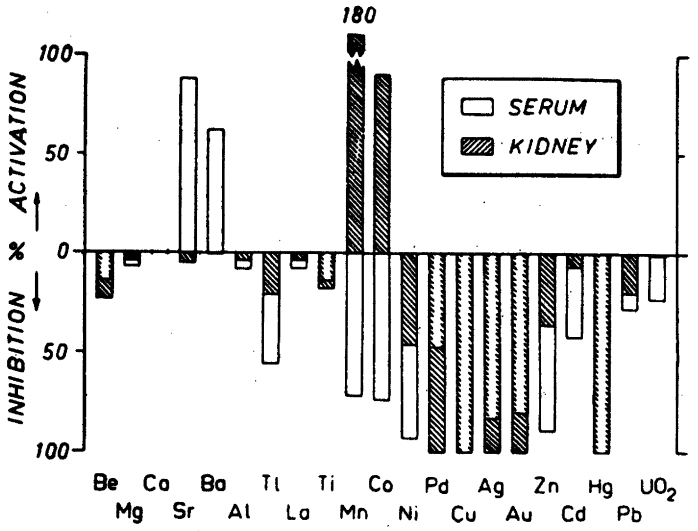

Fig. 1. Effect of various metallic ions on the phosphorylphosphatase activities of human serum and swine kidney (purified preparations). Substrate, 5.3 × 10^{-3} M tabun. Concentration of ions during activity determinations, 1.0 × 10^{-3} M. Time of incubation with ions, 60 minutes. Enzyme activity values corrected for non-enzymic hydrolysis rates, spontaneous substrate hydrolysis as well as non-enzymic hydrolysis catalysed by the metallic ions.

serum and rabbit kidney we found, among other things, that the two enzymes behave differently, the serum not being activated by Mn^{2+}, but inhibited instead [3]. The activation of the "DFPase" of swine kidney by Mn^{2+} was reported earlier by Mounter *et al.*[2], who later [4] confirmed our observation that the two enzymes show differences in respect to Mn^{2+} and other cations. The effect of a variety of ions has been investigated in this laboratory, and the present report deals with the results obtained with metallic ions.

METHODS AND MATERIAL

The Warburg manometric technique was used in determining enzyme activity [1]. All measurements were made at 25° C in 0.040 *M* sodium bicarbonate. The enzyme activity was expressed in b_{30} values. Tabun was used as substrate in most experiments; its concentration in the reaction mixture (total volume, 2.00 ml) was 5.3 × 10^{-3} *M*. DFP of the same molar concentration was also employed in some comparative studies.

The enzyme preparations used were Fraction IV-1 of human postpartum serum and a purified preparation of swine kidney. The latter was prepared according to the method described by Mounter *et al.*[3] for "DFPase". Both these phosphorylphosphatase preparations were dialysed free of ions against distilled water and then diluted to a proper concentration for enzyme activity determinations with bicarbonate solution.

The chlorides of the metals were used, except $AgNO_3$, $Pb(NO_3)_2$, $TlNO_3$, and UO_2SO_4. In preliminary testing of the effects of the metallic ions, the salt concentration in the reaction mixture during activity determination was 1.0 × 10^{-3} *M*. The enzymes were incubated 60 minutes with the ions, unless otherwise stated, before the substrate was added.

RESULTS

Effects of various metallic ions on the enzymic hydrolysis of tabun. The effects of metallic ions, the concentration of which was 1.0×10^{-3} *M* during enzyme activity determination, were studied using purified preparations of phosphorylphosphatases of human serum and swine kidney. The results are shown in Fig. 1. The serum enzyme was activated by Sr^{2+}, and Ba^{2+}; these ions had practically no effect on the kidney phosphorylphosphatase. Mn^{2+} and Co^{2+} were strong activators of the kidney enzyme, the former being particularly effective; the serum enzyme, on the other hand, was inhibited by these cations. Strong inhibitors of both types of phosphorylphosphatases were Pd^{2+}, Cu^{2+}, Ag^{+}, and Hg^{2+}. In addition, Ni^{2+} and Zn^{2+} were strong inhibitors of the serum phosphatase and moderate inhibitors of the kidney enzyme. Fe^{2+} (not inserted in Fig. 1) is a weak inhibitor of the serum phosphatase; this ion, however, is not suitable to study in bicarbonate systems, and no further work has therefore been carried out with Fe^{2+}.

Activation and inhibition by certain metallic ions as function of ion concentration. Those ions, which were found to be strong activators or inhibitors of the phosphorylphosphatases, were used in more detailed studies of the effects as a function of ion concentration. The results are shown in Fig. 2 for the serum enzyme and in Fig. 3 for the kidney enzyme. The *activating* ions gave optimum effect when present in a concentration of 10^{-3} *M*. In the presence of still higher concentrations of these ions the enzyme activity was slightly decreased or remained constant. It was especially noticeable that Mg^{2+} and Ca^{2+} had practi-

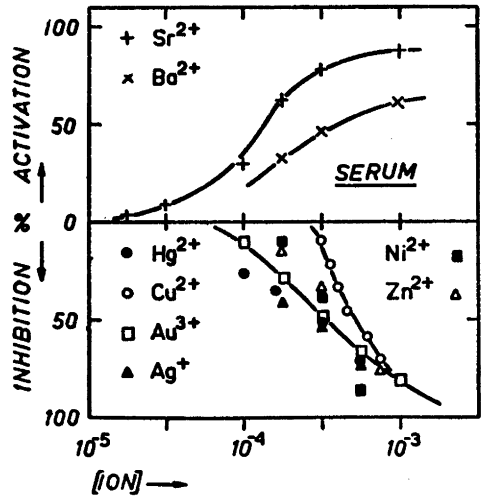

Fig. 2. Effect of metallic ions on the enzymic hydrolysis of tabun by human serum phosphorylphosphatase as function of molar ion concentration. Values corrected for non-enzymic hydrolysis (cf. text to Fig. 1). The values obtained for Mn^{2+} are found in Fig. 4. The pI_{50} values (— log molar ion concentration giving 50 % inhibition of enzyme activity) of the inhibiting ions are summarized in Table 1.

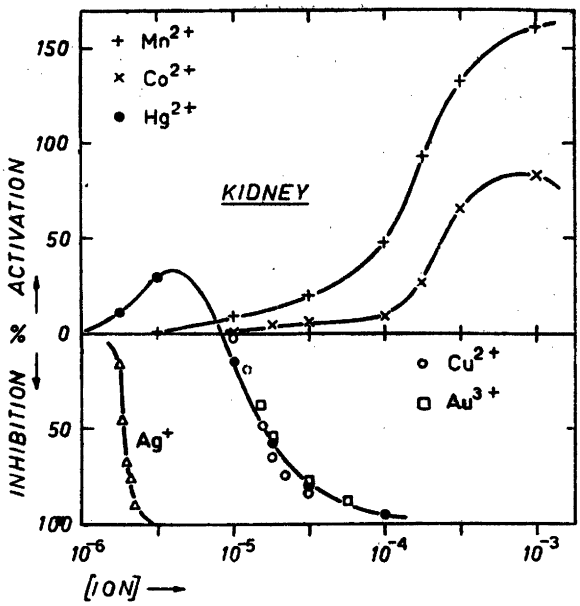

Fig. 3. Effect of metallic ions on the enzymic hydrolysis of tabun by swine kidney phosphorylphosphatase as function of molar ion concentration. Values corrected for non-enzymic hydrolysis (cf. text to Fig. 1). The pI_{50} values of the inhibiting ions are summarized in Table 1.

cally no effect on either of the two enzymes studied. This is in contrast to the observation made by Mazur [5] who recommended Mg^{2+} or Ca^{2+} as activators in the enzymic hydrolysis of DFP by liver extracts.

Those ions which are strong *inhibitors* of the phosphorylphosphatases were found to be equally active for each of the enzymes studied. That ion concentration which gives 50 % inhibition of the enzymic hydrolysis of tabun, expressed in pI_{50} values (— log molar ion concentration), is approximately the same for various ions: 3.2—3.4 for the serum enzyme and 4.7 for the kidney enzyme.

The effects of Hg^{2+} and Ag^{+} on the kidney phosphorylphosphatase are especially interesting. Mercuric ions, being equally active as inhibitors as, for instance, Cu^{2+} and Au^{3+}, increase the enzyme activity when present in low concentrations. Such a Hg^{2+}-activating effect was observed by Mazur [5] for the hydrolysis of DFP by liver, but could not be confirmed by Mounter *et al.*[2] with a purified preparation similar to that used in the present investigations. The inhibiting effect of Ag^{+} on the kidney phosphorylphosphatase is very striking; 50 % inhibition was obtained by 2.81×10^{-6} *M* Ag^{+} (pI_{50} 5.55), which is a concentration approximately ten times lower than those of Cu^{2+}, Au^{3+}, and Hg^{2+} giving the same degree (50 %) of inhibition. The pI_{50} values are summarized in Table 1.

Effects of metallic ions on the non-enzymic hydrolysis of tabun in the absence of phosphorylphosphatase. It was recently demonstrated by Wagner-Jauregg *et al.*[6] that certain metallic ions themselves catalyse the hydrolysis of DFP

Table 1. Effect of metallic ions (10^{-3} M) on the non-enzymic hydrolysis of tabun (expressed in b_{30} values), and molar ion concentration (in pI_{50} values) giving 50 % inhibition of the phosphorylphosphatase activities of purified preparations of human serum and swine kidney. a: activation. Cf. Fig. 1.

Metal ion	No enzyme present	pI_{50}	
		Kidney	Serum
Be^{2+}	8	<2	<2
Mg^{2+}	0	—	—
Ca^{2+}	4	—	—
Sr^{2+}	3.5	a	—
Ba^{2+}	2	a	—
Al^{3+}	4	—	—
Tl^{+}	5.5	3	<2
La^{3+}	5	—	—
Ti^{3+}	3.5	<2	<2
Mn^{2+}	0	3.33	a
Co^{2+}	17	3.20	a
Ni^{2+}	18	3.26	<3
Pd^{2+}	23 *	3.3	4.3
Cu^{2+}	30	3.18	4.70
Ag^{+}	21.5	3.43	5.55
Au^{3+}	23	3.28	4.66
Zn^{2+}	13	3.22	<3
Cd^{2+}	6.5	<3	—
Hg^{2+}	4	3.30	4.66
Pb^{2+}	4	<2	<2
UO_2^{2+}	6	<2	—

* 5×10^{-4} *M* Pd^{2+}.

and other organophosphorus compounds. Cu^{2+} is particularly active in such reactions, especially when present as chelates with dipyridyl, histidine, *etc.* The metallic ions used in the present studies, were also investigated for their effects on the non-enzymic hydrolysis of tabun. Amongst the ions studied the following are the most active catalysts: Cu^{2+}, $Pd^{2+} > Au^{3+} > Ag^{+} > Ni^{2+} > Co^{2+} > Zn^{2+}$ (Table 1). It is interesting to note that Mn^{2+} is without any effect on the non-enzymic hydrolysis.

Comparison between the effects of ions on the enzymic hydrolysis of DFP and tabun. In the investigations reported by Mounter *et al.*[2] the enzymic hydrolysis of DFP by swine kidney was found to be activated by Mn^{2+}. In Fig. 4 the effects of Mn^{2+} on the DFP hydrolysis catalysed by the phosphorylphosphatases of serum and kidney are compared with the results obtained with tabun as substrate. As far as the kidney enzyme is concerned this ion increases the activity for both substrates. The percentage activation of the DFP hydrolysis, however, is much more striking than that of the tabun hydrolysis. For serum phosphorylphosphatase, Mn^{2+} is an inhibitor in both the DFP and tabun reactions. The strongest inhibitor of the cations, Ag^{+}, studied with tabun as substrate is also a very potent inhibitor of the DFP hydrolysis by the kidney enzyme.

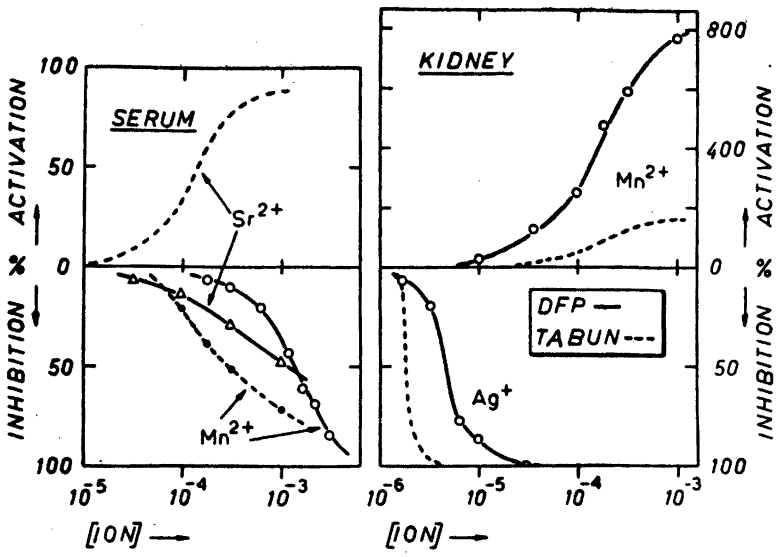

Fig. 4. Comparison between the effects of ions on the enzymic hydrolysis of tabun and DFP. Data referring to tabun (dotted lines) are taken from Figs. 2 and 3.

As mentioned above, Sr^{2+} is an activator of the enzymic hydrolysis of tabun by serum phosphorylphosphatase. However, when DFP is used as a substrate for this enzyme, Sr^{2+} acts as an inhibitor. In view of this observation one could surmise that two different enzymes are responsible for the hydrolysis of the two substrates. It was, however, demonstrated in a previous paper by the present authors [1] that DFP and tabun are hydrolysed by the same enzyme of the preparation of human serum used (Fraction IV—1) (see further "Discussion" below). As far as the kidney phosphorylphosphatase is concerned the results reported in the present paper are consistent with the view that DFP and tabun are hydrolysed by the same enzyme.

Activation and inhibition by metallic ions as time reactions. The enzyme activation by a metallic ion cannot be entirely electrostatic (there is no requirement for activation energy in such reactions) if the interaction between the metal and the protein is a time-reaction. Examples of such time-reactions, in which active enzymes are formed, have been described elsewhere [7]. In the present series of studies on the effects of ions on phosphorylphosphatases, the enzymes were incubated with certain metallic ions for various periods of time before the substrate (tabun) was added to the incubation mixture. The enzyme activity values obtained are shown in Fig. 5. It is clear from these results that there is a fundamental difference between the mechanism of the action of metallic ions on the two phosphorylphosphatases. Both the activation (by Mn^{2+}) and the inhibition (by Ag^+) of the kidney enzyme are time-reactions, in sharp contrast to the activation (by Sr^{2+}) and the inhibition (by Mn^{2+}) of the serum enzyme, which are independent of time. It will be noted that the

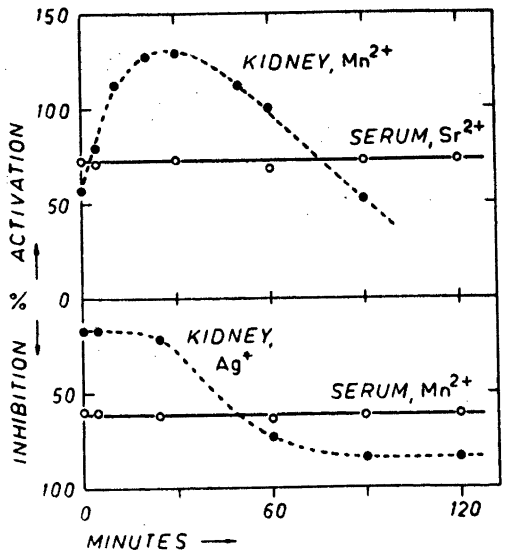

Fig. 5. Activation and inhibition of phosphorylphosphatases incubated for various periods of time with metallic ions. Molar concentrations of the metallic ions during incubation: 1.25 × 10⁻³ M Mn^{2+}, 1.25 × 10⁻³ M Sr^{2+}, and 3.75 × 10⁻³ M Ag^{+}; N.B. the ion concentration during activity determinations are 1.25 times less than those during incubation.

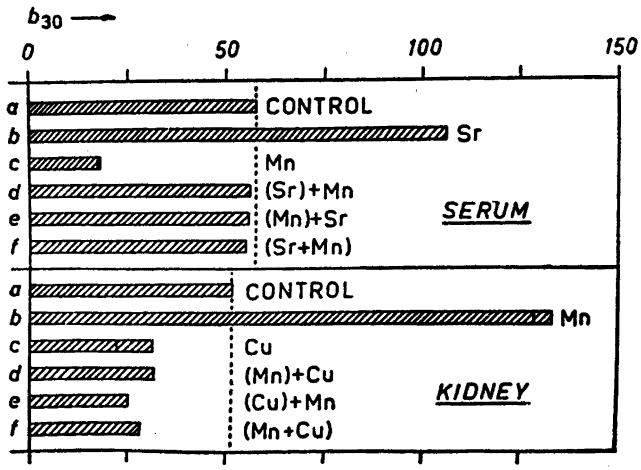

Fig. 6. Competition between activating and inhibiting metallic ions. The enzymes (activities: a) were incubated 30 and 60 minutes with either ion (b, activator; c, inhibitor), 30 minutes with the activator and then a further 30 minutes with the inhibitor (d), 30 minutes with the inhibitor and then a further 30 minutes with the activator (e), and 60 minutes with a mixture of activator and inhibitor.

"zero" values were obtained by adding the substrate a few seconds *before* the addition of the metallic ion.

Competition between activating and inhibiting metallic ions. Activating and inhibiting ions were found to compete with each other for active groups of the phosphorylphosphatase molecules. The results showing this phenomenon are demonstrated in Fig. 6 by a preliminary experiment. The activating effect of an ion is depressed by an inhibiting ion even if the enzyme was incubated with the activator 30 minutes before the inhibitor was added. This competition phenomenon will be studied in more detail before any discussion of its meaning is given.

Table 2. Effect of dialysis of enzyme preparations incubated with metallic ions. Enzyme activity was determined "before" and "after" dialysis and expressed in b_{30} values.

		Serum		Kidney	
		Metal	b_{30}	Metal	b_{30}
Control		—	85	—	48
Activator	Before	Sr^{2+}	145.5	Mn^{2+}	118
	After		85		32
Control		—	116.5	—	41
Inhibitor	Before	Mn^{2+}	52	Ag^{+}	4.5
	After		101		5

Effect of dialysis of enzyme preparations incubated with metallic ions. The enzyme preparations were incubated with activating and inhibiting metallic ions respectively and the mixtures dialysed against distilled water. The phosphorylphosphatase activities of the dialysed solutions were then determined and compared with those of the non-dialysed ones. The results obtained are recorded in Table 2. The effect of the activator (Sr^{2+}) as well as of the inhibitor (Mn^{2+}) of the serum enzyme is reversed by dialysis. The inhibition of the kidney enzyme, on the other hand, cannot be reversed by this procedure. The effect of dialysis on the Mn^{2+} activated is most interesting and unexpected. The activity of the activated enzyme is depressed markedly after dialysis and is even lower than the activity of the original non-treated enzyme. In one experiment (not demonstrated in Table 2) the enzyme, activated with Mn^{2+} to about 150 %, had lost after dialysis 67 % of its original activity. Before more experiments have been carried out, no explanation of this phenomenon will be attempted.

DISCUSSION

There are a number of ways in which a metal can be visualized as influencing the activity of an enzyme. Apart from the role of metals in the iron and copper oxidation-reduction enzymes, in which the usual function of the protein is to keep the metal in solution and to stabilize the state of lower valence, there may be a primary effect of the metal on the enzyme protein or co-operative effect

of metal and protein. These mechanisms of the action of metals can be exemplified by several well-known enzyme systems [7-10].

Let us first consider the mode of action of a metallic ion on the enzyme. By combining with an enzyme molecule, the metallic ion gives the complex a net charge which is obviously different from the original charge of the protein. At the same time the affinity of the enzyme for the substrate may be changed. This effect can be entirely electrostatic; there is no requirement of activation energy in such reactions and the effect is independent of the time of interaction between metal and protein. In addition to modifying the charge, the metal can change the configuration of the enzyme protein, *e.g.*, by reacting with —SH groups when these groups are necessary for enzyme activity. It may be pointed out that in these instances of metal-enzyme interaction the change in enzyme activity (it may be activation or inhibition) does not necessarily imply that the metal is involved directly in the formation of an enzyme-substrate complex. In the present study on the effects of metallic ions on the human serum phosphorylphosphatase, the activation by Sr^{2+} (tabun as substrate) and the inhibition by Mn^{2+} (and other ions as well) might be explained by this latter idea. The interaction between metallic ion and enzyme in these cases is independent of time (Fig. 5) and the metal most probably does not act as a mediator (Sr^{2+}) nor does it prevent the formation of an intermediate complex (Mn^{2+}, *etc.*). In fact, the observation made that the DFP hydrolysis by this enzyme is inhibited instead of being activated by Sr^{2+} makes it likely that the metal does not act as a mediator, but reacts at a site of the enzyme molecule which is responsible for the splitting of the P—CN (in tabun) and P—F linkages in the substrate molecules. The Sr^{2+}-protein complex favours this reaction for tabun but inhibits it for DFP.

For many metal-activated enzyme reactions the mechanism of activation postulated is that the metal acts as a mediator (bridge) between enzyme and substrate. It has been suggested that in this type of metal activation a chelate is formed between the metallic ion and the substrate, this chelate-formation being necessary for the enzyme activity. One of the arguments against this hypothesis is that those metals (*e.g.*, Mn^{2+} and Mg^{2+}) which possess weak chelating activity are commonly occurring activators of hydrolytic enzyme reactions. An alternative role for the metallic ion in such reactions was recently postulated by Klotz and LohMing [11]. According to this idea, the metallic ion favours the formation of the activated enzyme-substrate complex by co-ordinating with both enzyme and substrate and by increasing the local concentration of OH^- thus speeding up the hydrolysis. This idea seems to be applicable to Mn^{2+} and Co^{2+} activation of the hydrolysis of tabun by the kidney phosphorylphosphatase. The substituents [$(CH_3)_2N$—, C_2H_5O—, and CN—] of tabun, determining the specificity of the enzyme, are assumed to be bound directly to the enzyme (not to the metal in form of a chelate). The inhibiting ions (Cu^{2+}, Au^{3+}, Hg^{2+}, *etc.*) also form complexes with the enzyme protein, but these complexes prevent the substrate from reacting with the enzyme. In this respect, Ag^+ is particularly active.

Various opinions have been formed to explain the fact that metals have different (activating as well as inhibiting) effects on one particular enzyme activity and also that one metal can activate one enzyme and inhibit another.

As demonstrated in the present paper Mn^{2+} is an example of a metallic ion which can activate one enzyme and inhibit another and the explanation might be that there are two different mechanisms for enzyme activity which are influenced in different ways by the metallic ion. As far as the Mn^{2+} activation of the kidney phosphorylphosphatase and other enzymes is concerned and taking into account the fact that Mg^{2+} is without any effect on the enzymes studied by us, in contrast to the similar effect of Mg^{2+} and Mn^{2+} on other enzym systems, it may be useful to compare the electron configuration of Mn with that of Mg. Magnesium has a filled outer shell (3 *s*) and manganese a half-filled 3 *d* shell (5 electrons instead of 10). Manganese is the only divalent ion with this electron structure which may explain the different effects of Mn^{2+} and Mg^{2+}. It may also explain the instances when Mn^{2+} behaves like Co^{2+} which has a partly filled 3*d* shell (7 electrons).

Such an explanation does not seem to be valid for the effect of metallic ions on the serum phosphorylphosphatase. This is particularly true for the Sr^{2+} activation of this enzyme which to the authors' knowledge is the only known enzyme activation by this ion. Whatever the explanations of the effects studied may be, it is obvious that the activating effect of Sr^{2+} on the serum phosphorylphosphatase depends on a different mechanism to that for the Mn^{2+} activation of the kidney enzyme. From this we can conclude that the mechanisms of enzyme action of the serum phosphorylphosphatase and the kidney phosphorylphosphatase differ fundamentally.

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The Synthesis of D-Arabinose-1-^{14}C and D-Ribose-1-^{14}C

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A method for the synthesis of D-ribose-1-^{14}C and D-arabinose-1-^{14}C is described.

For certain biochemical investigations, we required some of the aldopentoses labelled with ^{14}C in the $C_{(1)}$ position, particularly D-ribose-1-^{14}C. To prepare these sugars we decided upon the cyanohydrin method, using D-erythrose as the starting material. When this work was undertaken Isbell *et al.*[1] had published methods for the synthesis of D-glucose-1-^{14}C and D-mannose-1-^{14}C from D-arabinose by the above method. Later when the present work was approaching completion they reported the synthesis of D-ribose-1-^{14}C and D-arabinose-1-^{14}C from D-erythrose [2]. Experimental details of their work has not yet been available to us.

The cyanohydrin synthesis of aldoses involves the addition of KCN to the one lower member of the homologous sugar series. The mixture of the two epimeric aldonic acids produced by hydrolysis of the nitriles is usually separated before the reduction of the lactones of the aldonic acids to the aldoses. Isbell *et al.*[1] separated the mixture of the D-gluconic and D-mannonic acids by fractional crystallization using the lactones and the barium salts. In the present case the reduction has been carried out with the mixture of the two epimers, and D-ribose and D-arabinose were finally separated by paper chromatography. For practical reasons, it was necessary to carry out the synthesis on a sufficiently small scale to enable the separation of the sugars on a few sheets of filter paper. For the sake of convenience no purification of the intermediates was attempted, although this resulted in a lower over all yield. Approx. 0.3 millimole K^{14}CN with an activity of 1 mC was used in the present synthesis. The high specific activity of the cyanide made it possible to dilute considerably the radioactive sugars with inactive material since only a specific activity of approx. 1 μC per milligram was desired.

Isbell *et al.* discovered, when synthesising labelled glucose and mannose from D-arabinose, that the ratio of the epimeric aldonic acids depended upon whether the cyanohydrin reaction was acid catalyzed (predominantly mannonic acid) or base catalyzed (predominantly gluconic acid). We attempted similar conditions in the cyanohydrin synthesis starting from D-erythrose, but in both

cases arabonic acid was formed in a high proportion. To obtain a mixture with a higher proportion of the ribonic acid we decided to induce epimerization of the arabonic acid by the method of Fischer [3,4] which involves heating of the latter with pyridine. Unfortunately the degree of epimerization was not as high as hoped for, only approx. 20 %.

EXPERIMENTAL

Preparation of D-*erythrose.* The D-erythrose employed was prepared from 4,6-diethylidene-D-glucose by the method of Rappaport and Hassid [5]. The product was a slightly yellow syrup and had $[\alpha]_D^{20} -19.8°$ (water, *c* 6.86).

Addition of $K^{14}CN$ *to* D-*erythrose.* The method used was a modification of that reported by Isbell *et al.*[1] Potassium hydroxide (11 mg) and $K^{14}CN$ (20.6 mg, 1 mC, containing 9 mg potassium hydroxide) were dissolved in water (3 ml) in a 50 ml flask fitted with a long neck and a quick fit stopper. The mixture was frozen in a mixture of acetone and solid carbon dioxide. D-Erythrose (80 mg) dissolved in water (1 ml) was added and subsequently solid carbon dioxide (160 mg). The stopper was kept in position and the mixture allowed to thaw. When melted the stopper was removed for a moment to release the pressure. The flask was kept at 0° C for 20 hours and then at room temperature for two days. The mixture was then heated to 50° C while bubbling air through the solution. To trap any radioactive HCN, the escaping air was passed through a solution of KOH. The reaction mixture was then concentrated to dryness *in vacuo* in the solid state. The residue was dissolved in water (1 ml) and ethanol (15 ml) added and the mixture put aside at 0° C for 4 days. Crystalline plates of the potassium salts of the aldonic acids together with a small amount of syrupy material had precipitated. The supernatant was easily decanted off and the crystals washed three times with alcohol (10 ml each time) and finally three times with ether (10 ml each time). The last traces of ether were removed in a desiccator.

Epimerization of arabonic acid. The crystalline mixture of the potassium salts of the aldonic acids was dissolved in water (30 ml) and passed slowly through a column of Amberlite IR-120(H) (7 ml wet resin, hydrogen form) to convert the salt to the free acids. The column was washed with water until negligible activity appeared in the eluate. The solution was concentrated to dryness in the solid state *in vacuo*. The residual syrup was dissolved in a small volume of pyridine (0.07 ml) and transferred to a pyrex tube (diameter 1.5 cm, length 10 cm). The flask was washed out four times with water (0.25 ml each time). The tube was sealed and heated at 130° C for two days. The dark content of the tube was diluted with water (10 ml), passed through a column of Amberlite IR-120(H) (4 ml wet resin, hydrogen form) and the column washed until negligible activity appeared in the eluate. The slight colour of the eluate was removed by treatment with charcoal. The filtrate was evaporated to dryness in the solid state *in vacuo* to yield a straw coloured syrup.

Lactonization of the aldonic acids. To induce lactonization the mixture of the aldonic acids was heated at 90° C for one hour *in vacuo* (0.01 – 0.001 mm Hg).

Reduction of the lactones with sodium amalgam. To ensure a suitable pH during the reduction we have used the mixture of oxalic acid and sodium oxalate introduced by Isbell and collaborators [1] for the reduction of the lactones of gluconic and mannonic acids. As suggested by these workers we also used a 5 % sodium amalgam. We encountered, however, difficulties in preparing a sodium amalgam which would give us a constant yield of the pentoses. Judging from trial experiments using inactive material the yield of the pentoses seemed to depend upon the quality of the amalgam. In one reduction we would obtain the usual yield of the sugars and with a new batch of sodium amalgam practically nothing. It seems to us that an amalgam with poor quality is obtained if the temperature during its preparation is too high. In our experience the temperature must not be so high as to give a dark surface on the amalgam. Thus when preparing 5 % sodium amalgam we proceeded as follows*: A 3 % amalgam was prepared by adding

* We thank Professor M. Stacey F.R.S. for helpful discussions on this point.

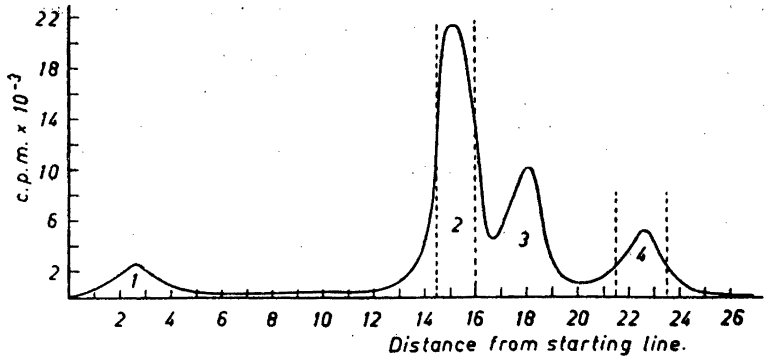

Fig. 1. The distribution of radioactivity along the paper chromatogram. Peak 2 and 4 are arabinose and ribose respectively.

sodium to the pure mercury, the amalgam was subsequently slightly heated and further sodium added. The mixture was then heated under stirring until just melted and poured into oil.

The reduction was carried out in a tube (diameter 2 cm, length 10 cm) fitted with a glass stopper which contained a small hole for the hydrogen to escape. The tube was immersed in a mixture of ice and water. The mixture of the lactones was dissolved in icecold water (3.5 ml) and transferred to the tube. Oxalic acid (117 mg) and sodium oxalate (133 mg) were added. Sodium amalgam (800 mg, 5 %) was then added and the mixture shaken for three hours in the cooling mixture, and left at room temperature over night. To remove all ions the reaction mixture was passed through a small coloumn of Amberlite IR-120(H) (2.5 ml wet resin) and subsequently through a column of Amberlite IR-4B (OH) (2.5 ml wet resin). The coloumns were washed with water until negligible activity was present in the eluate. The amounts of pentoses was determined in the eluate using the colourimetric method of Euler and Hahn [6]. The eluate contained 11.4 mg and 9.5 mg when calculated as arabinose and ribose respectively. This corresponded to a yield of approx. 25 % when calculated from the amount of K^{14}CN used.

Chromatographic isolation of D-*ribose-1-*^{14}*C and* D-*arabinose-1-*^{14}*C.* The eluate containing the two pentoses was concentrated to dryness *in vacuo* in the frozen state. The residue was dissolved in a small volume of water (0.4 ml). An aliquot (0.1 ml) was put on a sheet (25 cm × 55 cm) of Whatman paper No. 3 in a band of length 15 cm and width 2 mm. On both sides of the band was put a mixture of inactive ribose and arabinose as reference compounds. The paper chromatogram was run for three days in the top layer of a mixture of water-butanol-ethanol-ammonia (49:45:5:1, v/v/v/v). The reference runs were developed with aniline hydrogene phthalate and used as a guide when locating the positions of the radioactive sugars. A very narrow strip (0.5 cm) was cut out of that part of the paper which contained the radioactive run, cut transversely in small segments (0.5 cm width) and the activity of the segments determined using a thin window Geiger Müller tube. The distribution of the activity along the chromatogram is seen on Fig. 1. Peak 2 is arabinose and peak 4 is ribose. The other two peaks are unidentified. The areas on the chromatograms containing the radioactive sugars were cut out, these are indicated between the dotted lines in Fig. 1. It was necessary to run three more chromatograms to separate the remaining of the mixtures of the pentoses. The radioactive sugar strips were eluted with water and the solutions concentrated to dryness in vacuum in the frozen state, yielding small amounts of white material. The amounts of the pentoses were determined according to the method of Euler and Hahn [6]. The yields were:

D-arabinose-1-^{14}C 6 mg and D-ribose-1-^{14}C 1.8 mg.

The purity of the sugars was tested by running paper chromatograms in different solvent mixtures and examining the radioactivity along the chromatograms as described

above. The arabinose was homogeneous in the following solvent systems: butanol-acetic acid-water (4:1:5, v/v/v) and phenol saturated with water. It had an activity of 3.24×10^6 c.p.m. per milligram. Ribose when examined in the butanol acetic acid solvent contained 5—6 % of impurities. The radioactive ribose was therefore further purified by paper chromatography in this solvent system and the ribose eluted again from the chromatogram. It was only necessary to run one chromatogram to achieve this. The purified ribose-1-^{14}C was homogeneous in the phenol water solvent system. It had an activity of 3.07×10^6 c. p. m. per milligram. The sugars were plated directly and counted using the thin window Geiger Müller tube.

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Studies of Absorption Spectra

IX. The Spectra of Cobalt(II) Complexes

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The spectra of hexammine and tris(ethylenediamine) cobalt(II) ions were measured taking special precaution against the formation of the highly coloured oxidation products. The mono- and diammine spectra were found by use of Bjerrum's equilibrium data. The spectra of several octahedral cobalt(II) complexes are compared, and the displacements of band maxima discussed. The spectrum of the tetrachloro complex is shown to agree with the crystal field theory if a tetrahedral configuration is assumed. All the cobalt(II) complexes which have been considered here are magnetically normal, *i. e.* $S = 3/2$ in the ground state. The strong bands are then attributed to transitions $S = 3/2 \rightarrow 3/2$ while the weak bands originate from transitions $S = 3/2 \rightarrow 1/2$. Further the spectra of complexes with two different ligands are discussed and connected with the configuration and existence of geometrical isomers.

The crystal field theory, primarily set forth in the papers of Bethe [1] and van Vleck and his co-workers [2-4] has been successfully applied [5-9] to the spectra of the transition elements. The present paper compares the measured spectra of different cobalt(II) compounds with the theoretical predictions offered by the crystal field theory. Regular octahedral complexes with cubic symmetry such as the hexaaquo ion $[Co(H_2O)_6]^{++}$, the hexammine ion $[Co(NH_3)_6]^{++}$ and the tris(ethylenediamine) ion $[Co\ en_3]^{++}$ are of particular interest.

The two latter complexes were investigated by Roberts and Field [10]. The absorption spectrum they report for $[Co\ en_3]^{++}$ does not lend itself to a closer identification of the absorption bands. Thus it was observed that the steep absorption below 400 mμ can be reduced much below what is found by Roberts and Field when air oxidation is completely prevented. The operations were carried out in an atmosphere of nitrogen, and the technique used was to dissolve solid $CoSO_4$, $7H_2O$ in an oxygen free aqueous solution of the amine. Fig. 1 shows the spectra of 0.005 *M* $CoSO_4$ in 12 *M* NH_3 and in 0.5 *M* en, containing thus the nearly pure hexammine complexes, according to the equilibrium measurements by Bjerrum [11]. It is apparent from the curves that there is practically no absorption in the near ultraviolet.

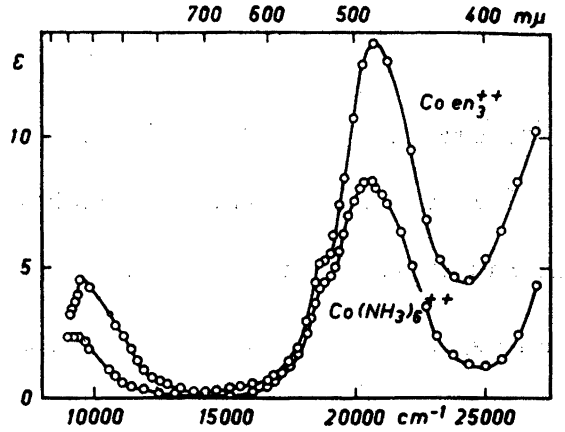

Fig. 1. Absorption spectra of hexaammine and tris(ethylenediamine)cobalt(II) ions. 0.005 M $CoSO_4$ (AnalaR) in 12 M NH_3 and 0.5 M en, respectively, at 25° C.

The strongly absorbing oxidation products seem to be one individual compound in each case, since the spectra of the solutions with different degrees of slight oxidation deviate linearly from the spectra of the hexa-complexes. The compounds formed are probably bi-nuclear peroxo complexes which in the course of some hours undergo changes to mono-nuclear cobalt(III) complexes. These latter complexes are known to be much less absorbing than the intermediate peroxo complexes. The reaction mechanism for the oxidation of Co(II) amine complexes is now being investigated in this laboratory.

The method for determining the spectra of the individual nickel (II) ammonia complexes [11] has here been applied to the two first complexes of cobalt(II), $[Co(NH_3)(H_2O)_5]^{++}$ and $[Co(NH_3)_2(H_2O)_4]^{++}$, respectively. Fig. 2 gives the two spectra, measured in 2 *M* NH_4NO_3 as well as the spectrum of $[Co(H_2O)_6]^{++}$ in the same salt solution.

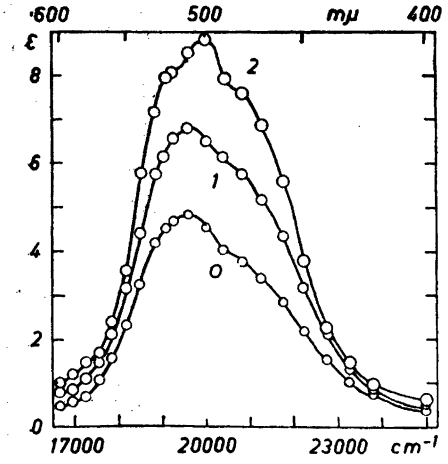

Fig. 2. Resolved absorption spectra of $Co(NH_3)_n^{++}$ in 2 M NH_4NO_3 at t = 25° C for n = 0, 1 and 2. Determined from the equilibria constants given by Bjerrum [11] for this salt medium and temperature.

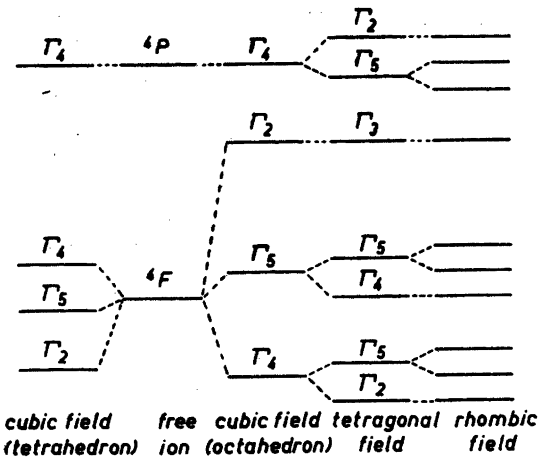

Fig. 3. Energy levels of ^4P *and* ^4F (d^7) *in crystal fields of different symmetry.*

The Co(II) ion has the electron configuration $1s^2 2s^2 2p^6 3s^2 3p^6 3d^7$. Thus two terms with $S = 3/2$ are possible, *viz.* the ground state ^4F and the ^4P term which is placed 15 400 cm^{-1} above the ^4F level [12]. The splittings of these two terms in crystal fields of various symmetry are shown schematically in Fig. 3. The notation is the nomenclature of Bethe [1].

The orbital degeneracy is

	Γ_1	Γ_2	Γ_3	Γ_4	Γ_5
cubic	1	1	2	3	3
tetragonal	1	1	1	1	2

It is seen that a tetragonal field removes nearly all the orbital degeneracy. The effect of a rhombic field is to do away with all the orbital degeneracy.

For Co(II) complexes with cubic symmetry the theory then predicts three bands, originating from transitions between the ground level and the three excited states all with $S = 3/2$. Further it is seen that the absorption spectrum for a regular octahedral complex is different from that of a regular tetrahedral Co(II) complex. The crystal symmetry is the same, but the energies of the states are different in the two cases.

An important theorem set forth by Jahn and Teller [13] states that in a stable molecule all orbit degeneracy is removed from the ground state. This rule can be applied to complex ions as follows: Consider a cubic configuration of the ligands around the metal ion. The energy then required to move the ligands a little from their equilibrium positions in the potential holes is of second order in the displacement and therefore small compared to the first

order energy gain produced by a possible accompanying splitting of the ground level. This continues until the ligands have arranged themselves in such a way that the ground state is unable to split up any further; the orbit degeneration of this level is then one. This rule predicts nothing about the order of magnitude of the distortion of the ligands from cubic symmetry, it merely states that there is a distortion. In some cases, however, the effect may perhaps be clearly seen as for example in the complexes of Cu(II). A regular octahedral Cu(II) complex with cubic symmetry has the ground state Γ_3. In a tetragonal field this level will split up into two new states [6], both once degenerated. The Jahn-Teller rule thus predicts that it is a tetragonal structure which is stable for Cu(II) complexes, as in fact demonstrated [5].

Fig. 3 shows that if the Jahn-Teller rule is to be obeyed octahedral Co(II) complexes must necessarily be of a tetragonal or of a rhombic structure. That this is the case is strongly supported by magnetic evidence [3]. However, the splittings of the states due to the superimposed rhombic field is only a few hundred cm^{-1}, and consequently these splittings only affect the absorption spectra to a very slight extent. It is therefore sufficient to consider the splittings due to the predominant cubic field in order to explain the absorption spectrum. On the other hand it is seen that it is possible to have regular tetrahedral Co(II) complexes, as the ground state here is a cubic Γ_2 level which is once degenerated.

OCTAHEDRAL Co(II) COMPLEXES

The level order is here $^4F(\Gamma_4)$, $^4F(\Gamma_5)$, $^4F(\Gamma_2)$ and $^4P(\Gamma_4)$. The infrared band of $[Co(H_2O)_6]^{++}$ measured by Dreisch and Trommer [14] at 8 000 cm^{-1} has been identified [7] as the first transition $^4F(\Gamma_4)$—$^4F(\Gamma_5)$. Similar bands were found for $[Co(NH_3)_6]^{++}$ at 9 000 cm^{-1} and for [Co en_3]$^{++}$ at 9 800 cm^{-1} (Fig. 1).

The theory [9] predicts three bands placed at $\nu_1 = 4/5\ (E_1—E_2)$ cm^{-1} $\nu_2 = 9/5\ (E_1—E_2)$ cm^{-1} and $\nu_3 = 3/5\ (E_1—E_2) + 15\,400$ cm^{-1} respectively. The parameter $(E_1—E_2)$ is the energy difference between the two possible levels of a single d electron in a crystal field of cubic symmetry.

To obtain the best agreement between the calculated and found absorption maxima we place $(E_1—E_2) = 9\,000$ cm^{-1} for the aquo-ion, $(E_1—E_2) = 10\,500$ cm^{-1} for the ammonia complex and $(E_1—E_2) = 11\,000$ cm^{-1} for the ethylenediamine complex (Table 1). This is in good agreement with the corresponding values for $(E_1—E_2)$ in Ni(II) complexes [8].

The two visible bands in $[Co(H_2O)_6]^{++}$ are then predicted to be situated at 16 200 cm^{-1} and 20 800 cm^{-1}, as seen in Table 1. The strongest band observed in the spectrum is a double band [15] with the centre of gravity at 20 200 cm^{-1}. This band is undoubtedly due to the transition $^4F(\Gamma_4) \rightarrow {}^4P(\Gamma_4)$, and is split up for similar reasons as the second band [8] of $[Ni(H_2O)_6]^{++}$. That the origin of this band is due to such a transition is further supported by the splitting found in the mono- and diammine cobalt(II) complexes, which have tetragonal symmetry. The low bands found [7] at 16 000 cm^{-1} in $[Co(H_2O)_6]^{++}$ and as shown above at 18 500 cm^{-1} in $[Co(NH_3)_6]^{++}$ and 18 700 cm^{-1} in [Co en_3]$^{++}$ are then identified as $^4F(\Gamma_4) \rightarrow {}^4F(\Gamma_2)$. The low intensity observed in these bands is probably connected with the fact that the state $^4F(\Gamma_2)$

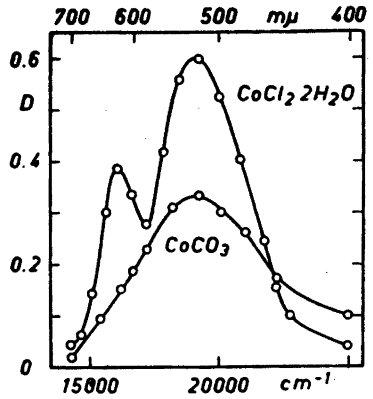

Fig. 4. Reflection spectra of cobalt(II) carbonate and cobalt(II) chloride dihydrate, measured on a Beckman DU spectrophotometer. t = 25° C.

has the orbital degeneracy = 1, while the much stronger combining state $^4P(\Gamma_4)$ is three times degenerate.

It may be shown that the symmetry and strength of the crystal field of an octahedral complex can be described in the following way: The sum of the contributions of the two ligands on each axis, we will call respectively $\sum(x)$, $\sum(y)$ and $\sum(z)$. The complex is said to have cubic symmetry when $\sum(x) = \sum(y) = \sum(z)$, and tetragonal symmetry when $\sum(x) = \sum(y) \neq \sum(z)$. Finally rhombic symmetry occurs when all three functions are different.

The crystal field states are alone determined by the set ($\sum(x)$, $\sum(y)$, $\sum(z)$). Thus all the complexes MA_5B, MA_4B_2 have tetragonal symmetry. The lowest possible symmetry, the rhombic, is represented by one of the geometrical isomers of MA_3B_3 (and of course in most complexes with three different ligands), while the other isomer of MA_3B_3 has cubic symmetry.

The influence on the metal ion of other crystal fields than cubic ones are considered by the authors [5,6,8,17] and by Orgel [16]. It turns out that the monoammine and the *cis*- and *trans*-diammine of Co(II) are each expected to exhibit two bands instead of the pure cubic band $^4F(\Gamma_4) \rightarrow {}^4P(\Gamma_4)$. The band $^4F(\Gamma_4) \rightarrow {}^4F(\Gamma_2)$ cannot be split up by fields of lower symmetry, except when the excited levels of the ground state begin to be populated but this can only account for a few hundred cm^{-1} at room temperature. The splitting in *cis*-complexes is generally — ½ times the corresponding splitting in *trans*-complexes [17]. Thus in most cases the *cis*-complexes have only broad bands, in contrast to the *trans*-complexes where there is a distinct separation into two bands.

Table 1. Comparison of calculated and found absorption maxima for $[Co(H_2O)_6]^{++}$, $[Co(NH_3)_6]^{++}$ *and* $[Co\ en_3]^{++}$ *in* cm^{-1}.

$[Co(H_2O)_6]^{++}$	obs.	8 000	~16 000	20 200
	calc.	7 200	16 200	20 300
$[Co(NH_3)_6]^{++}$	obs.	9 000	18 500	21 100
	calc.	8 400	18 900	21 700
$[Co\ en_3]^{++}$	obs.	9 800	18 700	21 700
	calc.	8 800	19 800	22 000

The vivid colour changes of certain cobalt(II) salts from red to blue forms may be due to one of two different reasons. Either the bands of the hexaaquo ion are only slightly shifted, and can still be recognized, or the configuration of the complex is totally changed with the appropriate consequences for the bands. This difference in spectral change was empirically pointed out by Feitknecht [18] and Katzin and Gebert [19] and rationalized on the basis of crystal fields by Orgel [16].

The first case, the small changes of octahedral energy levels, is exemplified by many complexes with oxy-anions. Fig. 4 shows the reflection spectrum of cobalt(II) carbonate, measured on the Beckman D.U. The only maximum found is at 530 mμ, and the usual structure of the band with a shoulder towards blue is still observed. The maximum at 512 mμ of $[Co(H_2O)_6]^{++}$ is shifted to 540 mμ in concentrated sulphuric acid.[23] Solutions in phosphoric acid give similar changes especially at elevated temperatures, and Dr. N. Hofman-Bang has kindly pointed out to us that cobalt(II) salts in formamide show reversible changes of colour with temperature. In a spectroscope the blue colour is seen to be due to a broadening of the usual band and a continuous displacement towards the red. Similar observations can be made on solutions of cobalt(II) nitrate in glycerol.

The temperature effects mentioned above are due to shifts of equilibria as the weaker anion and organic solvate complexes are generally formed with absorption of heat. However, the spectrum of a definite complex, *e. g.* $[Co(H_2O)_6]^{++}$ undoubtedly changes with temperature. These changes correspond to stronger crystal fields at lower temperatures resulting in spectral shifts in the same direction as substitution of, *e. g.*, ammonia for water. This hypsochromic effect at low temperature can be demonstrated by pouring liquid air on solid cobalt(II) salts of the usual red colour, which changes to yellow-orange.

Analogous to this effect, the green nickel(II) salts turn sky-blue at liquid air temperature. The observed increased crystal field is probably due to the greater orientation of the ligands at the lower temperature.

TETRAHEDRAL Co(II) COMPLEXES

The absorption spectrum of cobalt(II) in concentrated hydrochloric acid has been measured by many authors, *e. g.* Formanek [20], Jones [21], Brode [22], Kiss and Gerendás [23], Dreisch and Trommer [14] and Katzin [24]. The spectrum as recorded on a Cary spectrophotometer is shown in Fig. 5. If the complex formed was octahedral with two molecules of water, the spectrum would show bathochromic effects and slightly tetragonal splitting, but would otherwise resemble the spectrum of the aquo ion. The purple solid $CoCl_2, 2H_2O$ has the environment two water in the *trans*-position and four chloride ions in the plane around the cobalt(II) ion according to X-ray measurements by Vajnstejn [25]. Here the reflection spectrum (Fig. 4) is also seen to resemble other octahedral Co(II) complexes. Contrary to this, the spectrum of a tetrahedral $CoCl_4^{--}$ can be described by a cubic crystal field with $(E_1—E_2)$ equal to $-4/9$ $(E_1—E_2)$ of an octahedral $CoCl_6^{-4}$ with the same distances. The inversion of the levels is indicated by the minus sign, the numerical factor 4/9 is given by one of the

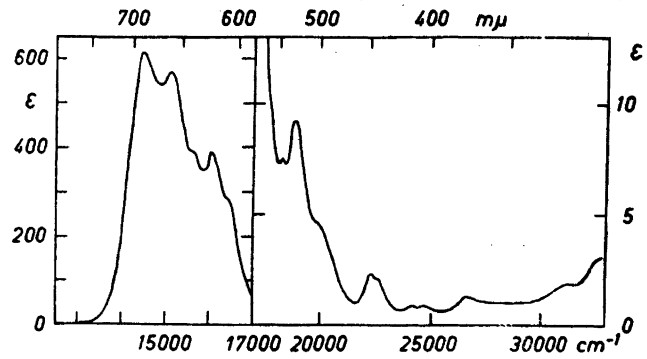

Fig. 5. Absorption spectrum of tetrachloro cobalt(II) ions (0.002 M *and 0.05* M $CoSO_4$ *in 12* M *HCl) measured on a Cary spectrophotometer.* t = 30° C.

authors[6]. Since $(E_1—E_2)$ in chloro-complexes is generally smaller than in aquo ions (compare Ti(III), V(III), Cr(III), Cu(II), *etc.*) one would expect $(E_2—E_1)$ to be somewhat smaller than 4 400 cm^{-1} in $CoCl_4^{--}$.

The band[15] at 6 300 cm^{-1} can be identified as a transition from the ground state $^4F(\Gamma_2)$ to $^4F(\Gamma_4)$ [see Fig. 3], giving 9/5 $(E_2—E_1)$ = 6 300 cm^{-1} or $(E_2—E_1)$ = 3 500 cm^{-1} in good accordance with the arguments given above. The other internal ^4F transition to Γ_5 must then be placed far in the infrared at ~ 3 500 cm^{-1}. The very intense band with a complex structure (five or six maxima) between 14 300 and 16 400 cm^{-1} is then due to $^4F(\Gamma_2) \rightarrow {}^4P(\Gamma_4)$ transitions which should be placed at 15 400 + 6/5 · 3 500 = 19 600 cm^{-1}, supporting the idea that $(E_2—E_1)$ is even smaller than 3 500 cm^{-1}.

Except in the lanthanides and actinides this is the weakest crystal field ever observed. The spectrum must therefore reproduce the atomic energy levels[12] quite exactly. The ^2G level which is placed at 17 300 cm^{-1} in the free ion has the crystal field splittings[9] in cubic fields amounting to + 1/5, +1/35, + 1/10 and —13/70 $(E_1—E_2)$, around the centre of gravity. This corresponds to a total splitting of 27/70 $(E_1—E_2)$ of the term. In $CoCl_4^{--}$ this is below 1 500 cm^{-1}. The bands at 18 300 and 18 800 cm^{-1} and probably also 19 800 cm^{-1} are due to ^2G, while the predicted centre of gravity is 17 300 + 6/5 $(E_2—E_1)$ = 21 500 cm^{-1}. The other weak bands are presumably due to the other doublet levels of Co^{++}, ^2H, ^2F, ^2D, *etc.* They continue down in the ultraviolet (Kiss and Gerendás[23] give the last at 34 700 cm^{-1}) corresponding to the latter doublets which are not known from atomic spectroscopy.

The bands are very narrow which is probably connected with the occurring weak crystal field, just as in the lanthanide and actinide spectra. The splitting of the $^4P(\Gamma_4)$ state is due to (L,S)-coupling effects, which are also quite large in the free ion[12]. Of the $(2S+1)(2L+1) = 4 \times 3 = 12$ independent states of ^4P the crystal field can only form six different levels, since the Kramers degeneracy cannot be removed. Of these six levels a pure cubic field should furthermore only present four, since two of the levels are four times degenerate on the basis of $(2S + 1)(2L + 1)$. It is highly improb-

able that the fine structure of the weak bands is due only to vibrational states (as in MnO_4^- or UO_2^{++}), as postulated by Brode [22] or Yamada and Tsuchida [26] since the intensities are so irregular, and no constant frequency difference can be found with certainty.

The intensity of the bands in $CoCl_4^{--}$ is anomalously large, compared with the other cobalt(II) complexes. While the transitions between states with $S = 3/2$ in most of the complexes have maximal molar extinction coefficients $\varepsilon_n \sim 10$ and the spin-forbidden transitions $S = 3/2 \rightarrow 1/2$ have $\varepsilon_n \sim 0.1$, the two different types of bands in $CoCl_4^{--}$ have $\varepsilon_n \sim 500$ and 5. This phenomenon is also encountered in chloro- and bromo-complexes of copper(II).

Simple crystal field considerations seem to be able to account reasonably for the experimental evidence, and it is possible to find some indications concerning the configuration of the various cobalt(II) complexes from their spectra. This again depends upon the perturbations from the ligands being strongly dependent on distances. The absorption spectrum of a given complex will then be determined mostly by the ligands in the first sphere, *i. e.*, the nearest environment of the metal ion.

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Some Problems of Intensity of Absorption Bands and Chemical Bonding

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The influences of different ligands on the wavenumber and intensity of the absorption bands of a given metal ion are compared. The intensities are connected with the intermixing of about one per cent odd functions in the even wavefunctions $[A]3d^n$. The other types of absorption bands in inorganic salts are classified. The difference between one and more times orbitally degenerate ground states in the cubic fields is shown to be relevant to complexity constants, activation energies and relative displacement of spectra of unsymmetrical complexes towards higher wavenumbers.

The crystal field theory of Bethe [1] has been applied to the absorption spectra of all the ions of the first transition group, first by Hartmann and Ilse [2,3], Orgel [4], and later by J. Bjerrum, Ballhausen and the author [5–11]. The agreement with the observed wavenumbers of the absorption band maxima is good, when reasonable values are assumed for the distances within the complex ions and the dipole moments or charges of the ligands. While the energy states are thus determined, the explanation of the transitions between the states has as yet not been fully given (the transition between states of the same electron configuration $[A]3d^n$ is forbidden by Laporte's rule and should have the probability 0). In the following discussion of this problem, some general remarks are first made on the energy states in different complexes of the same metal ion.

Already Fajans [12] developed the "spectrochemical series" of hypsochromic influence of different ligands *inter alia*, and Tsuchida [13] extended this concept further:

$$Br^- < Cl^- \lesssim OH^- < RCOO^- < F^- \lesssim H_2O < SCN^- < NH_3 < en \lesssim NO_2^- < o\text{-phen} < CN^- \quad (1)$$

When all six ligands are equal and placed at the same distances in an octahedral complex, the spectrochemical series (1) is easily explained as corresponding to increasing value of $(E_1—E_2)$ in a crystal field of cubic symmetry, where E_1

is the energy of a γ_3-electron and E_2 of a γ_5-electron [2,3,7]. It might seem curious that anions and neutral molecules all find their fixed places in (1), but it must be remembered that the point-dipoles used in calculations [2,5,6] are fictious quantities, only electrical charges occur in reality. In the neutral ligands, the negative part (distributed by polarization) is much nearer to the metal ion than the positive part, which thus has only a very small opposite influence on the crystal field perturbation, since it depends on R^{-5} or R^{-6} in most cases. R is the distance from the centre of the metal ion [2,6]. The real dipole moments of neutral ligands are undoubtedly somewhat smaller than the crystal field parameter μ caused by the strongly increased effects of the negative end of the dipole. The spectrochemical series (1) is not a direct measure of the affinity to the ligands, since the free energy changes depend on R^{-1} and R^{-2} potentials, which are much less dependent on distance. Therefore, anions, which are not strongly polarizable (as are CN^- and NO_2^-) have an anomalously early position in the series. But a quite paradoxical fact is the lower (E_1—E_2) value of OH^- than of H_2O. Probably, the three lone-pairs of electrons found in OH^- are not so effectively perturbing as the two lone-pairs of H_2O. Analogously, the latter molecule is not as effective as NH_3 with one lone-pair.

If a similar series as (1) is constructed of the intensities of the crystal field spectra, the anions will undoubtedly be placed at very late positions. Chloro- and bromo-complexes of copper(II) complexes have maximum molar extinction coefficients $\varepsilon_n = 100$ to 200 at $\sim 10\,000$ cm^{-1}, while $[Cu(H_2O)_6]^{++}$ has maximum at 12 600 cm^{-1} with $\varepsilon_n = 12$. Nitro-complexes of copper(II) measured by Fronæus [15], and cobalt(III) complexes among others by Basolo [16], show relatively very high intensities. In the preceding paper of this series [10], $CoCl_4^{--}$ was shown to have $\varepsilon_n \sim 500$ in ordinary and $\varepsilon_n \sim 5$ in ΔS-forbidden [7] bands, while in cobalt(II) complexes with neutral ligands the corresponding values are $\varepsilon_n \sim 5$ and $\varepsilon_n \sim 0.1$. In a paper to be later published, the dark-coloured bi-nuclear complex of titanium(III) and titanium(IV) in concentrated HCl will be discussed. It deceived Hartmann and Schläfer [14] to assume titanium(III) chloro-complexes alone to be ~ 10 times more absorbing than $[Ti(H_2O)_6]^{+++}$, while the real $[Ti(H_2O)_5Cl]^{++}$ is sky-blue. Since amine complexes are well known to be more absorbing than aquo ions, the intensity series can be written

$$H_2O < NH_3 < en \lesssim Cl^- < NO_2^- \lesssim Br^- \qquad (2)$$

with aquo ions being the least coloured complex of a given metal ion. Organic ligands give generally also increasing intensity, *viz.* alcohols and aliphatic amines, compared with H_2O and NH_3 respectively.

The theoretical explanation of intensity can in the author's opinion be found along the lines drawn by Broer, Gorter and Hoogschagen [17], who investigated the weak bands of lanthanide ions. The fundamental difficulty of providing internal d^n- or f^n-transitions with any positive probability is the following: Analogous to departure [18] from Russell-Saunders coupling, which intermix states of different L and S in such a way that only the vector sum J is preserved, the configuration interaction [18] may in the free ion intermix states of different electron configuration, but with the restriction that the

parity is the same. The parity, which thus is one of the most strictly defined quantum numbers of the free ion, is "even" or "odd", according to the sum of the values of l of the individual electrons in the system being even or odd. Laporte's rule for electric dipole radiation is: the transitions shall occur between states of opposite parity. Other radiation types, as electric quadrupole or magnetic dipole, give too small oscillator strengths [17] ($P \sim 10^{-8}$) for most lanthanide and all first-transition group bands, even though they allow transitions between states of the same parity. The oscillator strengths P can be found from the observed absorption spectra [19]

$$P = 4.32 \cdot 10^{-9} \int \varepsilon d\nu \simeq 4.6 \cdot 10^{-9} \; \varepsilon_0[\delta(+) + \delta(-)] \qquad (3)$$

ε_0 is the maximum extinction coefficient and δ (+) and δ (—) the two half-widths towards higher and lower wavenumbers. It is seen that for most normal bands [19] of the first transition group aquo ions, $P \sim 10^{-4}$. For the weak bands, where the total spin quantum number S changes 1 under the transition [7], $P \sim 10^{-6}$. The factor 10^{-2} is determined from the (L, S) coupling effects with intermixing of states with different S, but the same J as mentioned above.

Now, Broer, Gorter and Hoogschagen [17] pointed out that even the parity can be slightly intermixed by perturbations of crystal fields with no centre of symmetry. Also vibrations of the ligands can provide such an intermixing. In the lanthanides, the statically hemiedric perturbations are presumed [17] to be slightly more important than the vibrational. The intermixing of the wavefunctions can be formally expressed for the groundstate Ψ_1 and the excited state Ψ_2:

$$\begin{aligned} \Psi_1 &= a_1 \Psi_{even} + b_1 \Psi_{odd} \qquad (a_1^2 + b_1^2 = 1) \\ \Psi_2 &= a_2 \Psi_{even} + b_2 \Psi_{odd} \qquad (a_2^2 + b_2^2 = 1) \end{aligned} \qquad (4)$$

In the first transition group Ψ_{even} is represented by [A]$3d^n$ and Ψ_{odd} by [A]$3d^{n-1}4p$, while in the actinide ions the two types of Ψ are [Em]$5f^n$ and [Em]$5f^{n-1}6d$. In the latter case this treatment has given good agreement with the trend of observed intensities of the absorption bands in different actinides.

It must be remarked that the intermixing coefficients b_1^2 and b_2^2 are very small in most transition group complexes and that the meaning of "even" and "odd" is weakened, if this is not the case. Only the part $(a_1b_2 + a_2b_1)^2 \simeq (b_1 + b_2)^2$ will contribute to the oscillator strength of electric dipole radiation:

$$P \simeq \frac{1}{30} (b_1 + b_2)^2 \qquad (5)$$

The proportionality factor between 10^{-1} and 10^{-2} consists of the following factors: The wavenumber ν divided by the Rydberg constant 109 740 cm^{-1}; the "strength" of the transition, as defined by Condon and Shortley [18], divided by $e^2a_0^2$ (e is the electronic charge and a_0 the Bohr radius), and finally some statistical weights.

Since $P \sim 10^{-4}$, either the groundstate or the excited state or both (which is more reasonable) must be intermixed with odd functions to the extent of

$\sim 10^{-3}$. In the chloro- or ethylenediamine-complexes (see the series (2)), P has mostly increased to $\sim 10^{-3}$, and in the special case of $CoCl_4^{--}$ (where the tetrahedral configuration perhaps provides a stronger hemiedric perturbation than in octahedral complexes), $P \sim 10^{-2}$.

The intermixing of about one per cent odd functions in the complex ion states [A]$3d^n$ cannot be directly connected with the electron overlap from the ligands, which according to calculations of Craig, Maccoll, Nyholm, Orgel and Sutton [20] is recognized also to be $\sim 10^{-2}$. However, the assumption of one per cent covalency * may very well result in the same effects on intensity as the more atomic-spectroscopical picture given above. The most divergent point between the current theories of covalency in coordination chemistry and the crystal field theory is the assumption of the magnetic criterion. It seems unreasonable from the latter point of view to assume discontinuous appearance of considerable covalency in cobalt(III) complexes, whose two ordinary bands have $P \sim 10^{-3}$ as do magnetically normal complexes. Below, another criterion, the "robustness" of the complexes is discussed on the basis of the crystal field theory.

Empirically, it can be shown that the intensities of different bands of the same metal ion develop in slightly different ways. Let us consider [19] $Ni(H_2O)_6^{++}$ and Ni en_3^{++}. They give values of $(E_1—E_2) = 7\,600$ cm^{-1} and 10 500 cm^{-1} respectively, and their second and third bands have the transition probabilities:

		$Ni(H_2O)_6^{++}$	Ni en_3^{++}
$^3\Gamma_2 \rightarrow {}^3\Gamma_4(F)$	**	$P = 3.5 \cdot 10^{-5}$	$P = 10.2 \cdot 10^{-5}$
$^3\Gamma_2 \rightarrow {}^3\Gamma_4(P)$		$P = 7.2 \cdot 10^{-5}$	$P = 14.3 \cdot 10^{-5}$

If a similar calculation is performed with $Cr(H_2O)_6^{+++}$ and *** Cr en_3^{+++} which have respectively $(E_1—E_2) = 17\,400$ cm^{-1} and 21 600 cm^{-1}, one obtains:

	$Cr(H_2O)_6^{+++}$	Cr en_3^{+++}
$^4\Gamma_2 \rightarrow {}^4\Gamma_5(F)$	$P = 2.0 \cdot 10^{-4}$	$P = 11.1 \cdot 10^{-4}$
$^4\Gamma_2 \rightarrow {}^4\Gamma_4(F)$	$P = 3.0 \cdot 10^{-4}$	$P = 11.5 \cdot 10^{-4}$

Just as the series (1) is quantitatively expressed with the ratio between $(E_1—E_2)$ in the considered complex and in the aquo ion $((E_1—E_2)_{en}/)E_1—E_2)_{aq} = 1.38$ in nickel(II) and 1.24 in chromium(III) systems), the intensity series (2) seems to determine the intensity changes of the individual absorption band by change of ligand. For example in the two cases cited above P of the first band increases more than P of the second band. Generally, all chromium(III) complexes with relatively intense absorption have their first band highest contrary to the aquo ion.

* Intermixing with electron transfer states is supported by the higher intensities [7] of d^n-transitions in iron (III) compared to the isoelectronic manganese (II), or by copper(II) compared to nickel(II). The high intensity in anion complexes may be connected with the relatively low energy of the excited states with an extra electron in the metal ion.

** This band of the nickel(II)hexaaquo ion is presumably double, because the two states with $J = 2$ in ^3F and ^1D give an extraordinarily large intermediate coupling effect [18]. This causes intermixing of the state $^1\Gamma_3(D)$ predicted [11] at about 17 000 cm^{-1}.

*** Measurements by Mr. F. Woldbye in this laboratory.

These features cannot support the unfortunate distinction between the origin of the first and the second band, which confused Tsuchida's otherwise very useful classification [13]. Rather, Rabinowitch [21], Lindhard and Weigel [22] are correct in distinguishing between bands with $\varepsilon_n \sim 10$ due to energy states of the metal ion and bands with $\varepsilon_n \sim 10\,000$ due to electron transfer from the ligands to the metal ion. According to the modern theories, all the absorption bands of inorganic salts in the range between the vibrational bands in the infrared and the ionization bands in the far ultraviolet can be classified according to increasing perturbations of the environment:

f^n-transitions with nearly no chemical influence
($P \sim 10^{-6}$ in lanthanides and $\sim 10^{-4}$ in actinides)
$f^n \rightarrow f^{n-1}d$, broad bands with $P \sim 10^{-1}$ (see ref. 23, 24, 25)
d^n-transitions with strong influence of crystal fields
($P \sim 10^{-4}$ for $\Delta S = 0$, $P \sim 10^{-6}$ for $\Delta S = 1$ in the first transition group)
electron transfer spectra ($P \sim 10^{-1}$)
Electron transfer from ligands to metal ion or between different metal ions (*e. g.* some element in different oxidation states or with metallic bonding).

But the value $P \sim 10^{-4}$ assigned in this table to internal d^n-transitions is no absolute upper limit. In metal ions, strongly perturbed by the environments, it is possible to have $P \sim 10^{-1}$. Thus, it can be difficult to distinguish such bands from the electron transfer spectrum, as in the tris(*o*-phenanthroline) and tris(α,α'-dipyridyl) iron(II) complexes. The electron transfer spectrum may also have structure due to crystal fields, *e. g.* the tetrahedral CrO_4^{--} or MnO_4^- show two extremely strong bands. The groundstate contains only a very little of a 3d-electron wavefunction (perhaps causing the temperature-independent paramagnetism) while the excited state contains one 3d-electron, captured from the oxygen atoms, which can be either in the state γ_3 or γ_5. Recently, these ions were investigated by Wolfsberg and Helmholz [26] by the method of molecular orbitals. Empirically, it is known [27] that tetrahedral configurations give spectra with much more vibrational fine structure than complexes with octahedral configuration. The rare case of a weak electron transfer spectrum ($P \sim 10^{-4}$) is found in the bands of UO_2^{++} at 22 000 cm^{-1}, where the hexavalent uranium takes up a 5f-electron [25], while the strong ($P \sim 10^{-1}$) bands $\sim 40\,000$ cm^{-1} are due to formation of [Em]6d.

In the theory of chemical bonding, covalency has often been connected with the criterion of robustness, *i. e.* high activation energy (or extraordinarily low frequency factor). In the crystal field theory the standard state of reactivity is represented by ions with closed shells (Ca^{++}, Zn^{++}) whose ^1S-states are not influenced by crystal field perturbations. Magnetically normal d^5-systems (Mn^{++}, Fe^{+++}) have similar properties in their ^6S-states. Of the other d^n-systems in octahedral complexes, some have groundstates, which in crystal fields of cubic symmetry would be several times orbitally degenerate, *viz.* magnetically normal d^2, d^4, d^6, d^7 and d^9-systems, while the others have non-degenerate cubic groundstates, *viz.* d^3, d^8 and diamagnetic d^6-systems. It was

pointed out by Van Vleck [28] that the former type of complex with six equal ligands is not stable with cubic symmetry, but due to the Jahn-Teller effect must distort to a field of tetragonal (d^4, d^9) or even rhombic symmetry (d^1 can show "compressed" tetragonality [11] in the place of rhombic symmetry, having [10] $\Sigma(x) = \Sigma(y) < \Sigma(z)$). The two types of complex ion are referred to here as "distorted" and "regular" octahedral complexes.

The distorted complexes are seen to have low activation energies [29], sometimes even lower than the closed shell-configurations. In these cases, the distortion during the chemical exchange reaction is promoted by the tetragonal or rhombic splitting of the groundstate. However, in the regular complexes, the crystal field energy decrease is weakened during the distortion, because five (or seven loosely bound) ligands cannot produce as much perturbation as six ligands. The robust complexes with activation energy over 20 kcal/mole are all found in the regular group (*e. g.* nickel(II), cobalt(III), rhodium(III) and platinum(IV) complexes). Homogeneous reaction rates with cobalt(III) ammines and neutral ligands have as yet not been determined, but they probably all correspond to activation energies over 30 kcal/mole, while the cubic crystal field energy decrease [11] is $\sim$60 000 cm^{-1} = 85 kcal/mole. Electron transfer agents as carbon or palladium [30] catalyze the cobalt(III) reactions, probably because the labile cobalt(II) complexes are intermediately formed.

The consecutive formation constants of complexes $MA_{6-n}B_n$ and their absorption spectra are also widely different in the "distorted" and "regular" octahedral group. In the former group, characteristic [30] coordination numbers 2 and 4 can occur. While the latter is quantitatively supported [30] by copper(II) ammines, and qualitatively observed for chromium(II) complexes, evidence for the characteristic coordination number 2 is also indicated in the irregular behaviour of cobalt(II) ammines, where the existence range of the tri-ammine complex is anamalously narrow (Ref.[30], p. 287). In the regular group, the rest effects [30] are small except due to steric influences. Here, the absorption spectra are linear combinations [11] of $(1-\frac{n}{6})\,[MA_6] + \frac{n}{6}\,[MB_6]$, when the centre of gravity of tetragonal and rhombic split bands are considered. Contrary to this, the distorted group exhibits tetragonal splitting also of the groundstate and thus shows systematic displacement of the absorption spectrum towards higher wavenumbers by decreasing symmetry of the complex. This is exemplified in $[Cu(NH_3)_5H_2O]^{++}$, which is less pronounced tetragonal than $[Cu(NH_3)_4(H_2O)_2]^{++}$ and thus gives rise to the pentammine effect [5].

Hellwege [31] has investigated the selection rules in crystal fields and concluded that for octahedral complexes the transitions from Γ_4 to $\Gamma_1, \Gamma_3, \Gamma_4$ and Γ_5 and from Γ_5 to $\Gamma_2, \Gamma_3, \Gamma_4$ and Γ_5 and the reverse are the only possible ones for dipole radiation. Hellwege agrees with the present author that Laporte's rule is exactly valid in crystal fields, having a centre of symmetry (as have octahedral (O_h), but not tetrahedral (T_d) complexes). These selection rules are not entirely fulfilled by the the relative intensities of different transitions arising from the hemiedric potential. Thus, transitions from Γ_1 in diamagnetic cobalt(III) and from Γ_2 in nickel(II) and chromium(III) to both Γ_4 and Γ_5 have nearly the same probability. Therefore, only a rough proportionality to the orbital degeneracy number can be expected. Since the transi-

tion probabilities in fields of lower symmetry are not much higher than in the octahedral complexes, these cannot alone be important in determining the intensity of the bands. However, an increased intensity in tetrahedral and *cis*-MA_4B_2 complexes, due to hemiedric potentials, is observed in some cases.

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Surface-Chemical Studies on the Formation of Aluminium Soaps

I. The Interaction between Aluminium Ions and Rosin Acid Monolayers

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A continuously recording surface balance has been employed to study monomolecular layers of purified tall oil rosin acid on substrates of varying acidity and containing different amounts of potassium aluminium sulphate. The conditions promoting interaction between the monolayer substance and aluminium ions in the substrate have been investigated in detail in the presence of 2 *M* sodium chloride and without added salt. The monolayer properties of the aluminium rosinate formed by the interaction have been examined.

The conditions in which the interaction begins, in which it attains a wider scope and in which it leads to a definite endpoint are well-defined and reproducible. The aluminium rosinate formed has characteristic and constant monolayer properties.

In the year 1948 the present authors began a series of investigations with the object of defining the conditions which promote interaction between aluminium ions in aqueous solution and rosin acid spread in a monolayer on the surface of the solution. Up to the year mentioned most of the numerous investigations that had dealt with the formation of aluminium soaps had involved reactions in three-dimensional systems [1–11]. Only a few observations in this respect had been made using surface-chemical methods [12–14].

In our investigations we established [15] that by means of the surface balance technique it is possible to determine rapidly and in an elegant manner the conditions favouring such interactions. The same technique was then employed in a study of the interactions between aluminium ions and fatty acid monolayers [16,17]. These studies have later been extended to reactions between aluminium ions and the surfaces of solid rosin acid and solid fatty acids which were examined by another surface-chemical method, the measurement of contact angles [16,17]. This latter work confirmed, for instance, that the results obtained with the aid of the monolayer technique are of general significance. At approximately the same time as our first results were reported, Schulman

and coworkers published the results of their work on metal-monolayer interactions in aqueous systems in which they employed a similar technique to solve problems of a similar nature [18–21].

It is our intention to describe our investigations in a series of articles under the main title of this paper.

MATERIALS AND METHODS

The investigations described below were made with tall oil rosin acid because of the great technical importance of this material.

The rosin acid was prepared from crystalline tall oil rosin from Enso-Gutzeit Oy., Kotka, Finland. According to Juvonen [22], this product comprises 94.7 % rosin acids, 4.8 % unsaponifiables and 0.07 % ash; its melting range is 160—165° C. At Oy Keskuslaboratorio — Centrallaboratorium Ab, Helsingfors, the unsaponifiables and fatty acids were removed by extraction, the latter after esterification, and remaining rosin acids were recrystallized from ethanol. The melting point of this product was 166—169° and the mean equivalent weight 301. We then purified this product by the method of Aschan [3]. The substance was ground to a powder and allowed to remain for 20 hours under 80 % ethanol. The undissolved rosin acids were recrystallized twice from pure methanol and once from acetone. The rosin acid remaining melted in the range 168—174° C (corr.). The optical rotation $[\alpha]_D^{20}$ in absolute ethanol varied from −33° to −43° for different samples (measured with a Lippich polarimeter using a 2 dm tube, the concentration being about 0.01 g/ml).

Tall oil rosin acid is a mixture of different rosin acids [24]. According to Sanderman [25] it comprises about 50 % abietic acid, about 25 % dehydro-, dihydro- and tetrahydroabietic acids and about 25 % dextropimaric acid. Harva [26] has spectrophotometrically followed the purification of the Enso-Gutzeit tall oil rosin acid by recrystallization in the above-mentioned manner and found that the abietic acid content first rises to a level of 43 % but then remains fairly constant on further recrystallization.

Aschan and coworkers [27–29] established that the tall oil rosin acid mixture prepared in the above-mentioned manner behaves in many respects as a single rosin acid. This was to some extent confirmed by Harva [30] in respect of the physico-chemical properties of tall oil rosin soap in aqueous solution. Later studies in this laboratory with synthetic mixtures of pure rosin acids have shown that this is also the case in respect of the surface-chemical properties [31] and that the properties of the individual acids greatly resemble those of the tall oil rosin acid [32].

The rosin acid was dissolved in benzene (concentration, $2 \cdot 10^{18}$ molecules per ml). In order to prevent the oxidation of the abietic acid, the solution was stored in a glass-stoppered flask in a benzene atmosphere; fresh solutions were frequently prepared.

The rosin acid-benzene solution was added from an Agla micrometer syringe to the surface of the aqueous substrate in a Langmuir trough and the properties of the rosin acid monolayer formed investigated by the aid of an automatic, self-recording surface balance of Wilhelmy-Dervichian type [33,34] (improved by Anderson-Groth, Ställberg-Stenhagen and Stenhagen [35]). Compression of the monolayer was begun 2—3 minutes after the rosin acid had been spread on the surface; the rate of compression was 7—12 cm per minute.

When the effect of hydrogen ion concentration on the monolayer was studied, the pH values of the substrates were measured with a glass electrode and a saturated calomel reference electrode. The standard EMF was checked against phosphate buffer solutions.

TALL OIL ROSIN ACID MONOLAYERS AT DIFFERENT pH VALUES

Tall oil rosin acid monolayers give surface pressure-area curves (Fig. 1) that are of the same type as those obtained for the dextropimaric acids and tetrahydrodextropimaric acid by Harkins, Ries and Carman [36,37]. Both

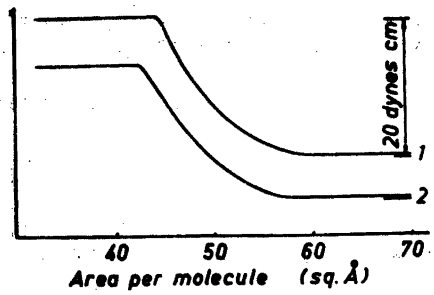

Fig. 1. π—A curves of tall oil rosin acid recorded on dilute hydrochloric acid (pH 3). 20° C. 1 The substrate contains no sodium chloride. 2 The substrate contains 2 moles of sodium chloride and 5 · 10⁻³ moles of acetic acid-acetate buffer per litre.

on pure water and on 2 *M* aqueous sodium chloride solution, the monolayers are of the liquid condensed type.

Fig. 2 shows the variation of certain characteristic points on the surface pressure-area curves with the pH of the substrate. A_0 denotes the area per molecule in the monolayer when the pressure first begins to rise, A_K the area per molecule at the point where the monolayer collapses and π_K the surface pressure at this point. (The collapse point is determined by extrapolation.) The mentioned quantities remain practically unaltered on acid substrates up to pH 4.2—4.6. Above this pH, the areas per molecule begin to decrease and the pressure to increase. It is obvious that at pH values below this limit, the rosin acid molecules are undissociated. The changes in the monolayer properties observed above this pH limit are evidently due to the inception of ionization in the monolayer. In Table 1 data characteristic of undissociated tall oil rosin acid monolayers are given.

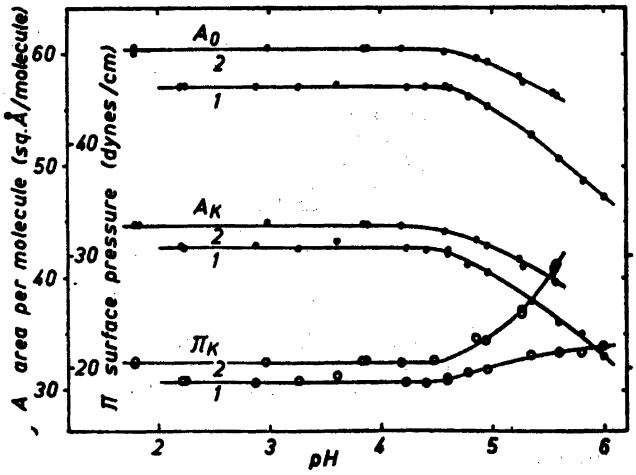

Fig. 2. The values of A_0, A_K and π_K for different pH values of the substrate. 20° C. 1 The substrate contains no sodium chloride. 2 The substrate contains 2 moles of sodium chloride and 5 · 10⁻³ moles of acetic acid-acetate buffer per litre.

Table 1. Characteristic data for undissociated tall oil rosin acid monolayers (below pH 4.2—4.6).

Substrate	A_0 Å² per molecule	A_K Å² per molecule	π_K dynes/cm
Water; pH adjusted with dilute HCl	57.1	42.7	19.4
2 *M* NaCl, buffered with $5 \cdot 10^{-2}$ *M* acetic acid-acetate	60.2	44.6	20.4

On the 2 *M* sodium chloride solution the molecules in a rosin acid monolayer take up an area about 2—3 Å² larger than on pure water. The pressure that the monolayer can withstand before it collapses is about 1 dyne/cm larger in the former case.

On pure water the ionization becomes evident in the monolayer properties in the pH range 4.4 to 4.6 and on 2 *M* sodium chloride in a slightly lower pH range between 4.2 and 4.5. The ionization results in an increase of the solubility of the rosin acid. It is, however, difficult to determine to what extent this increase of the solubility leads to a transfer of rosin acid ions from the monolayer into the substrate. It is also difficult to determine if the observed decrease in the area per molecule is caused only by this dissolving process. The fact that the collapse pressure increases slightly in this pH range shows that the mentioned area decrease is not solely attributable to the dissolution of the monolayer substance even in the case of pure water. On the 2 *M* sodium chloride substrate, in which the solubility of the monolayer substance is lower than in water, the decrease of the area per molecule is less and the increase in collapse pressure considerably larger than on pure water.

THE INTERACTION BETWEEN ALUMINIUM IONS AND TALL OIL ROSIN ACID MONOLAYERS ON 2 *M* SODIUM CHLORIDE

The surface balance method can be employed to define those conditions in which interaction between aluminium ions and rosin acid monolayers can and cannot take place. An interaction will lead to changes in the properties of the monolayer which will become evident in the form of the surface pressure-area curve [15]. This is shown by the curves in Fig. 3 which refer to tall oil rosin monolayers spread on substrates 2 *M* in sodium chloride and containing different concentrations of aluminium ions. In all cases the hydrogen ion concentration was regulated to pH 4.00 by means of an acetic acid-acetate buffer. Curve 3,0 is a recording taken when the substrate contained no aluminium ions, and curve 3,1 refers to a substrate containing $4.92 \cdot 10^{-5}$ mole of potassium aluminium sulphate per litre. No effect of aluminium ions is evident in the latter curve. As soon as the aluminium concentration is increased to $1.17 \cdot 10^{-4}$ *M* (curve 3,2), however, the curve undergoes a slight change; both A_0 and A_K decrease. These changes become more marked with increasing aluminium ion

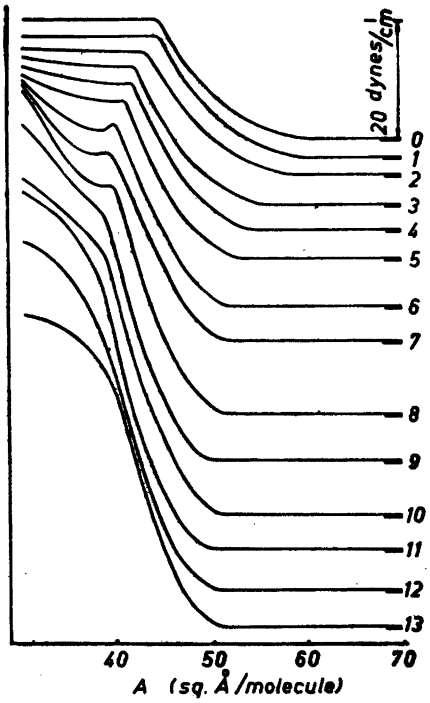

Fig. 3. π—A curves of tall oil rosin acid recorded on substrates of pH 4.00 containing 2 moles/l sodium chloride, 5 · 10⁻² moles/l acetic acid-acetate buffer and different potassium aluminium sulphate concentrations.

Curve No.	Al conc. moles/litre	Curve No.	Al conc. moles/litre
0	—	7	$5.00 \cdot 10^{-4}$
1	$4.92 \cdot 10^{-5}$	8	$6.14 \cdot 10^{-4}$
2	$1.17 \cdot 10^{-4}$	9	$9.22 \cdot 10^{-4}$
3	$1.78 \cdot 10^{-4}$	10	$1.05 \cdot 10^{-4}$
4	$2.21 \cdot 10^{-4}$	11	$1.17 \cdot 10^{-4}$
5	$3.08 \cdot 10^{-4}$	12	$1.86 \cdot 10^{-4}$
6	$4.32 \cdot 10^{-4}$	13	$5.02 \cdot 10^{-3}$

concentration; at the same time the curve shifts upwards and the collapse pressure π_K attains a higher value. The curve type passes through several intermediate stages from the original type to an altogether new type. This new type is fully developed in curve 3,12. When the aluminium ion concentration is increased further, this type remains constant; only the height of the curve diminishes slightly.

Values of A_0, A_K and π_K have been measured from a larger series of recorded curves of which those shown in Fig. 3 are representative. The variation

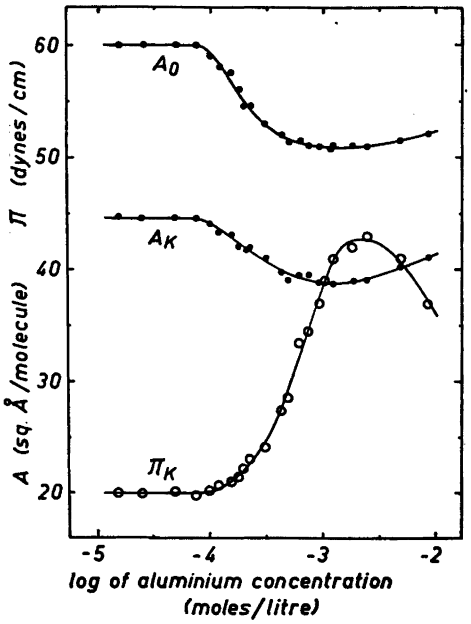

Fig. 4. The dependence of A_0, A_K *and* π_K *on the potassium aluminium sulphate concentration of the substrate at constant pH 4.00 · 20° C. The substrate contains 2 moles of sodium chloride and* $5 \cdot 10^{-2}$ *moles of acetic acid-acetate buffer per litre.*

of these quantities with the aluminium concentration is shown in Fig. 4. It is seen that when the pH of the substrate is held at 4.00, the tall oil rosin acid monolayer is unaffected by aluminium ions until the concentration of the latter exceeds $8.9 \cdot 10^{-5}$ *M*. Higher aluminium ion concentrations bring forth changes in the curves and hence in the structure of the monolayer itself. Each variation in the aluminium ion concentration effects definite reproducible changes in the monolayer properties. The previously mentioned new curve type is fully developed when the aluminium ion concentration is about $1.8 \cdot 10^{-3}$ *M*. The collapse pressure has increased from 20.4 dynes per cm to more than double this value, 42.6 dynes per cm. The interaction between the rosin acid molecules in the monolayer and the aluminium ions in the substrate gradually leads to an exchange of the monolayer substance from one of pure rosin acid to one composed of an aluminium rosinate, and this exchange seems to be complete at the aluminium ion concentration $1.8 \cdot 10^{-3}$ *M*. The new curve type is characteristic for monolayers composed of this aluminium rosinate. When the aluminium ion concentration is increased above the last-mentioned value, the aluminium rosinate layer undergoes further changes which become evident in the pressure-area curves: the collapse pressure π_K decreases again slowly and the areas per molecule, A_0 and A_K, increase gradually.

We have conducted a similar series of experiments in order to determine the effect of increasing the aluminium concentration at several pH-values. Some of the results are illustrated in the form of curves of the same type as in Fig. 4. Fig. 5 thus shows the effect on A_0, A_K and π_K of increasing aluminium ion concentration at pH 3.77, Fig. 6 at pH 4.32, Fig. 7 at pH 4.54, Fig. 8 at pH 5.00 and Fig. 9 at pH 5.60. In all cases the interaction between the

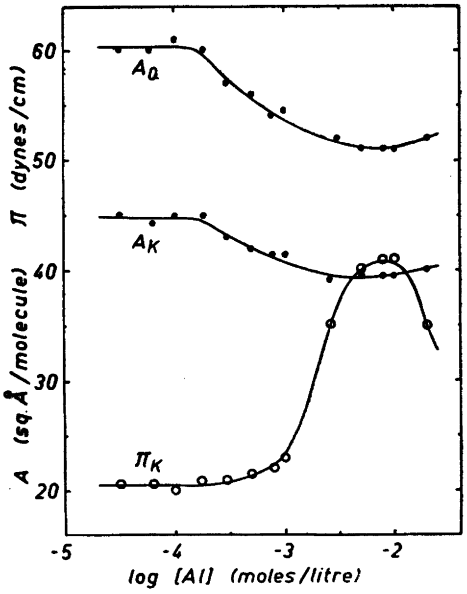

Fig. 5.

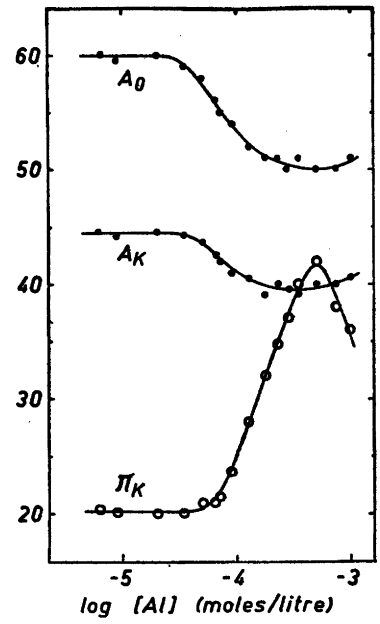

Fig. 6.

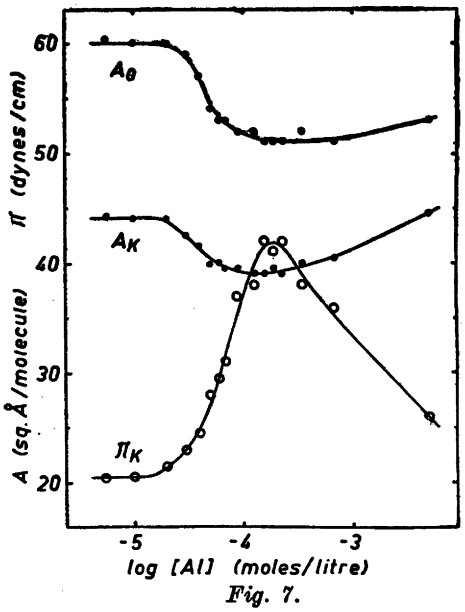

Fig. 7.

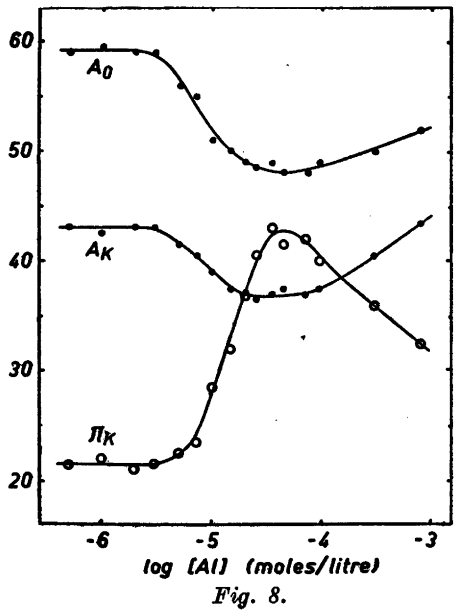

Fig. 8.

Figs. 5—9. The dependence of A_0, A_K and π_K on the potassium aluminium sulphate concentration at constant pH. The substrate contains 2 moles of sodium chloride and $5 \cdot 10^{-2}$ moles of acetic acid-acetate buffer per litre. Fig. 5 pH 3.77, Fig. 6 pH 4.32, Fig. 7 pH 4.54, Fig. 8 pH 5.00 and Fig. 9 pH 5.60.

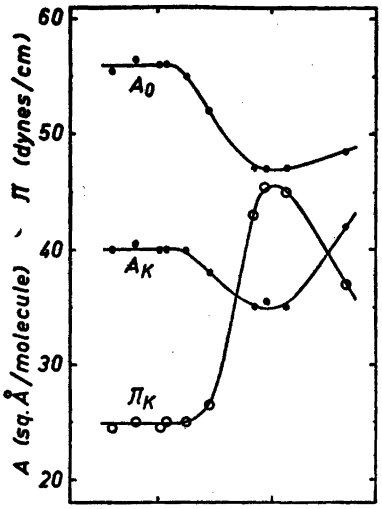

Fig. 9.

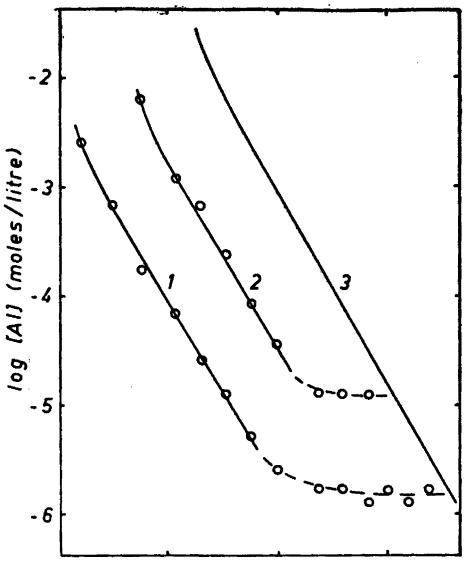

Fig. 10.

Fig. 10. The potassium aluminium sulphate concentrations and corresponding pH values of the substrate when the first signs of interaction between aluminium ions and the rosin acid (Curve 1) and when maximum interaction (Curve 2) is noted. Curve 3 gives the aluminium concentrations and pH values at which aluminium hydroxide precipitates in equilibrium conditions. 20° C. The $KAl(SO_4)_2$ solutions contained 2 moles of sodium chloride and $5 \cdot 10^{-2}$ moles of acetic acid-acetate buffer per litre.

aluminium ions of the substrate and the rosin acid molecules of the monolayer becomes evident in a similar manner. The lowest aluminium ion concentration at which this interaction occurs shifts, however, to lower values with increasing pH. The same applies to the lowest aluminium ion concentration at which the new curve type, the aluminium rosinate type, is fully developed. In all cases this curve type is fully developed at about the same aluminium concentration at which the value of collapse pressure attains its maximum and just before the area per molecule in the monolayer again begins to increase. By means of monolayer studies it thus is possible to determine the end-point of the reaction between aluminium ions and rosin acid which leads to the formation of a uniform monolayer of aluminium rosinate.

The results of the above-mentioned studies on the reaction between rosin acid and aluminium ions are summarized in the diagram of Fig. 10. Curve 1 gives the aluminium concentrations and corresponding pH values at which the interaction begins. Under the conditions to the left of this curve no effect of aluminium ions on the rosin acid monolayer is observed; to the right of the curve the interaction grows in scope until curve 2 is reached. In the conditions given by curve 2 the end point of the reaction is reached. The fully drawn curves give the conditions in which the reaction is independent of the time

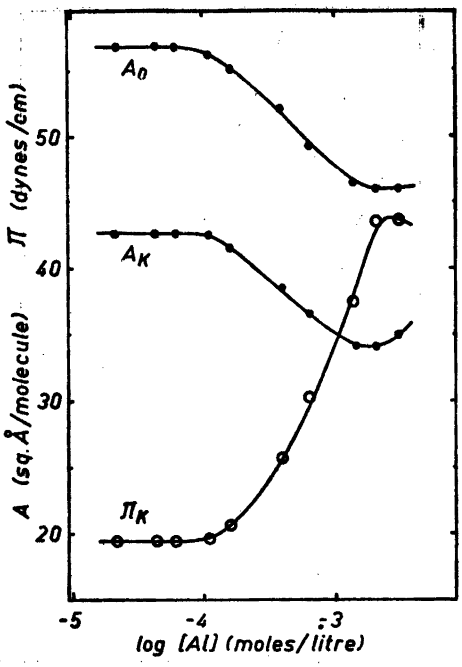

Fig. 11.

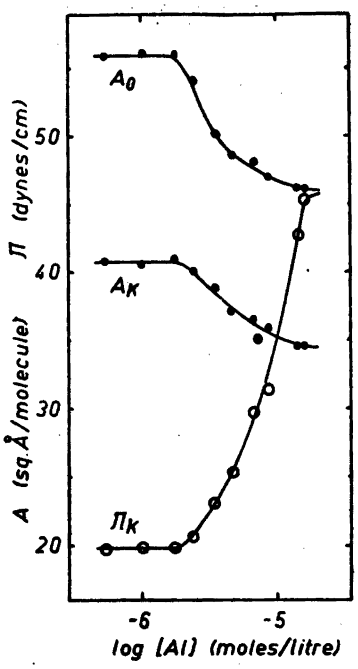

Fig. 12.

Figs. 11—12. The dependence of A_0, A_K and π_K on the potassium aluminium sulphate concentration at constant pH. 20° C. The substrate contained no added salts. Fig. 11 pH 4.00, Fig. 12 pH 5.00.

before compression is begun. At low aluminium concentrations, however, the interaction requires some time to attain equilibrium; the dotted parts of curves 1 and 2 apply to the time of reaction allowed in our experiments, *i.e.* a total reaction period of five minutes. If the aluminium ions are given a longer time to diffuse to the rosin acid monolayer, the first signs of an interaction will occur at somewhat lower aluminium ion concentrations and lower pH values. (This phenomenon will be discussed in more detail in a later publication of this series.)

The curve 3 in Fig. 10 gives the pH and aluminium concentration values at which aluminium hydroxide begins to precipitate under equilibrium conditions. This curve is based on investigations of potassium aluminium sulphate solutions of different pH values containing precipitated aluminium hydroxide. After 1—2 days the solution was separated from the precipitate by decantation and analysed for aluminium.

THE INTERACTION BETWEEN ALUMINIUM IONS AND TALL OIL ROSIN ACID MONOLAYERS ON WATER

The conditions promoting interaction between tall oil rosin acid monolayers spread on a substrate containing aluminium ions but no added sodium chloride

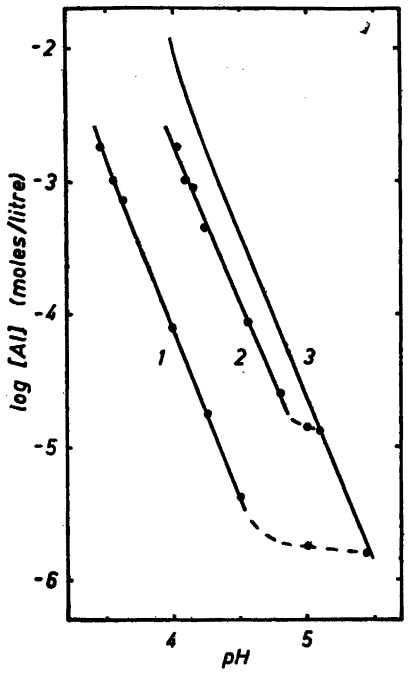

Fig. 13. The potassium aluminium sulphate concentrations and corresponding pH values of the substrate when the first signs of interaction between aluminium ions and the rosin acid (Curve 1) and when maximum interaction (Curve 2) is noted. Curve 3 gives the aluminium concentrations and pH values at which aluminium hydroxide precipitates in equilibrium conditions. 20° C. The $KAl(SO_4)_2$ solutions contained no added salts.

have been studied in a similar manner as above. The ionic strength of the substrate was only *ca.* 0.0001 in these experiments, compared with *ca.* 2.1 in the preceding experiments. The pH was adjusted with dilute HCl or NaOH.

The effect of the aluminium ions on the monolayer becomes evident in changes in the surface pressure-area curves similar to those described above. Figs. 11 and 12 show curves based on the observed changes which refer to the pH values 4.00 and 5.00, respectively.

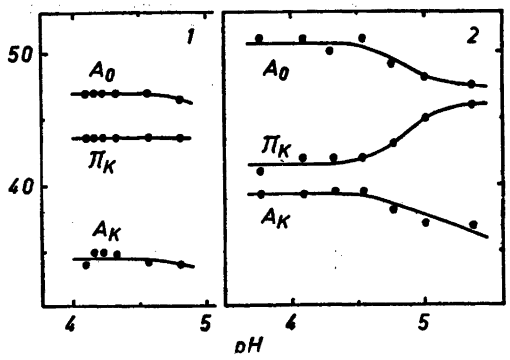

Fig. 14. The values of A_0, A_K and π_K of dibasic aluminium monorosinate monolayer at different pH values of the substrate. 20° C. 1 The substrate contains no added salts. 2 The substrate contains 2 moles/l sodium chloride and $5 \cdot 10^{-2}$ moles/l acetic acid-acetate buffer.

The experimental results can also in this case be summarized in a similar diagram as in Fig. 10. In this new diagram, Fig. 13, curve 1 shows the pH and aluminium concentration values up to which the properties of an rosin acid monolayer on water remain unchanged, curve 2 shows the conditions in which the end-point of the reaction between the aluminium ions and the rosin acid occurs and an uniform aluminium rosinate monolayer is formed. Curve 3 again gives the conditions where precipitation of aluminium hydroxide occurs. On water, especially when the aluminium concentration is low, the interaction begins and ends at lower pH values than when sodium chloride is present in the substrate. Also the precipitation of aluminium hydroxide takes place at lower aluminium concentrations and pH values. Owing to this, the curve 3 is situated at pH values only 0.2 unit higher than curve 2.

THE PROPERTIES OF THE ALUMINIUM ROSINATE MONOLAYER AT DIFFERENT pH VALUES

It has been possible to determine that when the new curve type, *i.e.* the curve for the aluminium rosinate monolayer, is fully developed, the monolayer is composed solely of dibasic aluminium monorosinate. The evidence proving this will be given in the following paper of this series.

The experimental data given in the preceding sections can be employed to determine how the properties of these aluminium monorosinate monolayers vary with the pH of the substrate (Fig. 14). On water the characteristic properties of the monorosinate monolayer remain unchanged up to a pH of about 4.7, but above this value the area per molecule decreases slowly. On 2 *M* sodium chloride, similar changes occur slightly earlier, at pH 4.5—4.6; also the collapse pressure increases. The data become erratic at pH 4.9 in the former case (water) and at pH 5.5 in the latter case (2 *M* NaCl); in this range the interaction with aluminium ions begins to depend on the time of reaction. Above pH 5.1 and 6.0, in the respective cases, it was not possible to obtain aluminium monorosinate monolayers giving well-defined pressure-area curves.

Table 2. Characteristic data for dibasic aluminium monorosinate monolayers (below pH 4.5).

Substrate	A_0 Å² per molecule	A_K Å² per molecule	π_K dynes/cm
Water; pH adjusted with dilute HCl	46.0	34.8	43.6
2 *M* NaCl, buffered with $5 \cdot 10^{-2}$ *M* acetic acid-acetate	50.7	39.3	41.6

Table 2 gives data which characterize the monolayers of dibasic aluminium monorosinate. These data have been obtained for monolayers formed on substrates with very different aluminium ion concentrations, from 10^{-5} *M*

up to 10^{-2} *M*. An increase in the sodium chloride content of the substrate leads to larger areas per molecule of the monolayer substance and causes the collapse pressure to fall slightly.

The deviations from the characteristic values given in Table 2 at higher pH values are observed at the same pH values at which a pure rosin acid monolayer begins to undergo ionization.

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Surface-Chemical Studies on the Formation of Aluminium Soaps

II. The Product of the Reaction between Aluminium Ions and Monolayers of Tall Oil Rosin Acid

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Monomolecular layers of purified tall oil rosin acid have been spread on substrates containing potassium aluminium sulphate under conditions in which interaction takes place between aluminium ions and the monolayer. The interacted layers have been skimmed from the surface and the content of aluminium, carbon and hydrogen in the collected substances has been determined.

The interaction leads to the formation of dibasic aluminium monorosinate. The existence of other aluminium rosinates in the monolayer has not been observed.

In the foregoing paper (Part I)[1] we described a series of surface balance measurements that were planned to define the conditions in which reaction takes place between a tall oil rosin acid monolayer and aluminium ions in the supporting substrate. The lowest aluminium concentration at which the reaction begins varies with the pH of the substrate and the reaction attains a definite end-point at a higher aluminium concentration which also depends on the substrate pH. This end-point is signalized by the fact that the changes in the monolayer properties effected by the reaction attain their maximum extent. In the following we shall describe the results of our investigation of the constitution of the monolayer substance.

EXPERIMENTAL

The isolation of the monolayer substance. In order to be able to conduct an analysis of the substance formed by the reaction between the tall oil rosin acid monolayer and aluminium ions it was necessary to collect a sufficient quantity of it. A monolayer of rosin acid was spread in the usual manner on an aqueous potassium aluminium sulphate solution of known concentration and pH. The monolayer substance was compressed to a very small area and removed with suction together with the smallest possible volume of the substrate. New rosin acid was again spread on the substrate and the monolayer substance compressed and removed; this procedure was repeated 200 to 500 times (for different

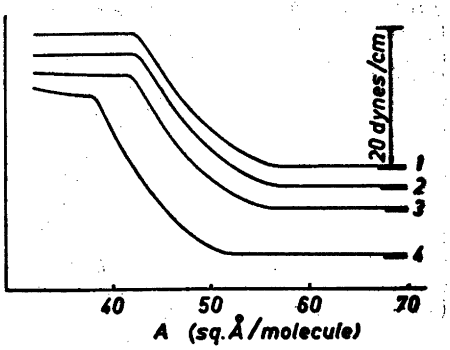

Fig. 1. π-A curves of tall oil rosin taken at different pH values for the determination of the aluminium ion concentration of the substrate. Curve 1 pH 4.12, 2 pH 4.20, 3 pH 4.28, 4 pH 4.39.

samples). After the monolayer substance had been skimmed off, it consisted of a white flocculent precipitate dispersed in some of the substrate solution. The precipitate was filtered, washed with a few millilitres of water and dried. The quantities of substance obtained in this way varied from 10 to 15 mg.

As the monolayer substance comprised an aluminium rosinate, the concentration of aluminium ions in the substrate was gradually reduced by the above-mentioned skimming procedure. This reduction was especially marked when the aluminium ion concentration was initially low. (In order to maintain the conditions as constant as possible, the substrate was renewed after several skimmings.)

It was established that also the pH of the substrate was altered when the surface layer was removed. In experiments in which the initial aluminium ion concentration was 1.4×10^{-5} *M*, the pH decreased from the value 5.20 to 5.08. In other experiments in which the aluminium ion concentration was 4×10^{-5} *M*, the pH diminished during 120 skimmings from 4.80 to 4.60 and 4.50 and in an experiment with the initial aluminium concentration 5.20×10^{-4} *M*, 240 skimmings lowered the pH from 4.25 to 4.18.

As we wished to know the constitution of the monolayer substance formed under definite conditions, the pH was checked and adjusted from time to time or the substrate was renewed after several skimmings. Since the changes were less marked when the aluminium content of the substrate was not too low, the aluminium concentration was held at 1×10^{-3} *M* in most of the experiments.

The analytical methods. Carbon and hydrogen in the isolated aluminium rosinate were determined by micro combustion analysis. (These analyses were performed by Mr. K. Salo at the Chemical Institute of the University of Helsingfors.)

The aluminium content was determined by a surface-chemical method developed for the purpose. The principle of the method is the following. 4—6 milligrams of the aluminium rosinate were ashed carefully in a platinum dish and the ash treated with 1—2 drops of concentrated sulfuric acid and some nitric acid. The dried aluminium sulphate was dissolved in water and diluted to a volume (about 1.3 litres) sufficient to fill the surface balance trough. A rosin acid monolayer was spread on this substrate and its pressure-area curve was recorded in the usual manner with the surface balance. The pH of the substrate was adjusted to known values and new pressure-area curves recorded for rosin acid at each pH value. It was found suitable to begin at a low pH value and increase it gradually. The recordings were continued until a definite change in the pressure-area curve revealed that the aluminium ions had begun to interact with the rosin acid monolayer. The data were employed to evaluate accurately the lowest pH value at which the interaction could be detected. Knowing this value of the pH, it was possible to read from curve 1 in Fig. 13 (in Part I of this series) the aluminium ion concentration of the substrate. This procedure could be repeated as many times as desired. The pH of the substrate was altered by adding sodium hydroxide or hydrochloric acid and measured with a valve potentiometer (Radiometer PHM 3) using glass and saturated calomel electrodes.

A number of pressure-area curves recorded during the course of our analyses are reproduced in Fig. 1. No effect of aluminium ions is noted in curve 1 (pH 4.12) and curve

2 (pH 4.20). In curve 3 (pH 4.28), the interaction with the aluminium ions has already become clearly evident; the area A_o has diminished from 57.0 to 56.2 Å^2 per molecule and the collapse pressure π_K has increased from 19.3 to 19.6 dynes per cm. In general, the pH range in which the effect of aluminium ions was first observed could be determined within ± 0.02 pH units. This permits the determination of the aluminium content of the solution with an accuracy of ± 0.038 units of the logarithm of the aluminium concentration, which corresponds to an accuracy of the analysis of ± 9 %. At first the analyses were conducted using tall oil rosin acid monolayers. Later one of us (Bruun) has employed tetrahydroabietic acid for the purpose, with an increase in accuracy (± 6 %).

Our aluminium analyses were usually conducted in the aluminium concentration range $3 \times 10^{-6} - 3 \times 10^{-5}$ *M*. The method of analysis cannot be employed with aluminium concentrations under 2×10^{-6} *M* (See Fig. 13 in part I of this series). It is hence necessary to have at least 0.07 mg aluminium for each analysis when our present surface balance (trough volume 1.3 litres) is used for recordings.

RESULTS

We were particularly interested to determine the constitution of the monolayer formed in the conditions where the surface balance studies indicated that the interaction of aluminium ions and the rosin acid monolayer attained a definite limit, *i.e.* under those conditions defined by curve 2 in Fig. 13 of the first paper of this series. The analyses 1—4 (see Table 1) relate to monolayer substance collected under conditions close to this curve. The material used in analyses 5 and 6 was collected under conditions to the right of this curve, but under conditions where no precipitation of aluminium hydroxide was yet possible. The conditions in which material for analyses 7, 8 and 9 was collected are represented by points in the region between curves 2 and 1, *i.e.* in the region where the monolayer has not yet been completely transformed into aluminium rosinate according to our surface balance studies in Part I. In Fig. 2 the mentioned diagram is reproduced and the conditions under which the different samples were collected are marked with circles.

The analyses of all samples of monolayer substance collected when the typical aluminium rosinate pressure-area curve had fully developed gave an

Table 1. Data defining the conditions in which the analysed monolayer substances were formed and the results of the analyses.

Analysis No.	Conditions for the formation of the monolayer substance		Number of skimmings	Quantity of monolayer substance in mg	The results of the analyses (%)			
	Al conc., *M*	pH			Al	C	H	Mean values
1	$1.3 \cdot 10^{-5}$	5.07	323	10.8	7.1	68.6	8.92	
2	$3.5 \cdot 10^{-5}$	4.67	240	12.4	7.6	66.8	8.87	
3	$9.4 \cdot 10^{-5}$	4.50	250	12.6	7.5	66.0	9.04	7.5 % Al
4	$5.1 \cdot 10^{-4}$	4.18	240	12.6	—	66.8	8.94	67.0 » C
5	$1 \cdot 10^{-3}$	4.26	360	13.6	7.8	—	—	8.94 » H
6	$1 \cdot 10^{-3}$	4.24	360	14.5	7.7	—	—	
7	$2.1 \cdot 10^{-5}$	4.71	480	13.3	5.4	68.6	8.92	
8	$1 \cdot 10^{-3}$	3.93	330	13.0	3.5	—	—	
9	$1 \cdot 10^{-3}$	3.88	220	10.0	3.3	—	—	

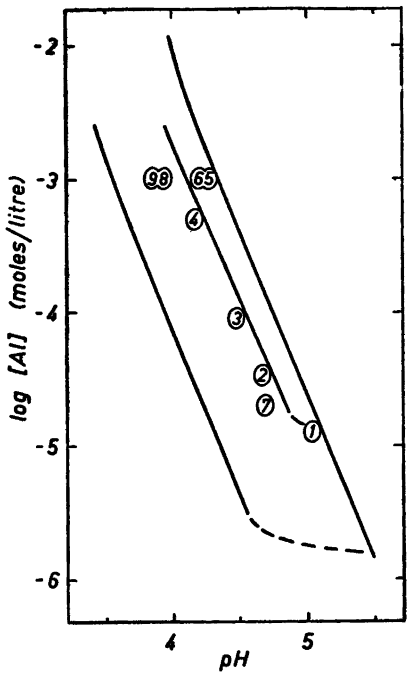

Fig. 2. Schematic diagram showing the conditions in which the monolayer samples were taken. The numbers refer to Table 1, the curves correspond to those in Fig. 13 of the first paper in this series.

aluminium content exceeding 7 %. The monolayer must hence have been converted into an aluminium monorosinate. The mean aluminium content, 7.5 %, corresponds closely to that calculated for a dibasic aluminium monorosinate, 7.44 % (Table 2). Also the carbon content is in agreement with that

Table 2. Theoretical aluminium, carbon and hydrogen contents for different aluminium rosinates (%).

Aluminium rosinate	Al	C	H
Dibasic aluminium monorosinate Ros Al—OH OH	7.44	66.3	8.56
Monobasic aluminium dirosinate Ros Al—Ros OH	4.17	74.3	9.13
Aluminium trirosinate Ros Al—Ros Ros	2.70	77.3	9.34

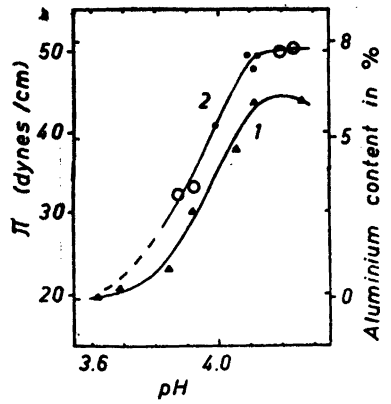

Fig. 3. The collapse pressure π_K (Curve 1) and the aluminium content (Curve 2) of tall oil rosin acid monolayers on substrates of different pH values containing $1 \cdot 10^{-3}$ mole/l of $KAl(SO_4)_2$. 20° C. (The pH values of the points on curve 2 were found by interpolation).

of aluminium monorosinate, but the hydrogen values deviate somewhat more and are between the values for the mono- and dirosinate.

Analyses 7—9 have given results which show that the monolayer substance in question corresponds to an aluminium rosinate of low aluminium content. The aluminium content decreases with the pH of the substrate on which the monolayer was formed *i.e.* the more the conditions differ from those yielding the aluminium rosinate pressure-area curve. It falls down to and below the aluminium content of monobasic aluminium dirosinate.

In order to give a clear picture of how the properties of the monolayer vary with its constitution, the curves in Fig. 3 are reproduced. Curve 1 in Fig. 3 shows the changes of the collapse pressure with pH on substrates containing 1×10^{-3} mole aluminium per litre. Curve 2 gives the aluminium content of the monolayer substance formed on solutions of the same composition. (The circles give experimentally determined aluminium values. In those cases where the monolayer substance has been collected on solutions of lower aluminium content than 1×10^{-3} *M*, the corresponding pH value on the substrate mentioned has been interpolated from the curve 2 of Fig. 13 in Part I.) The point

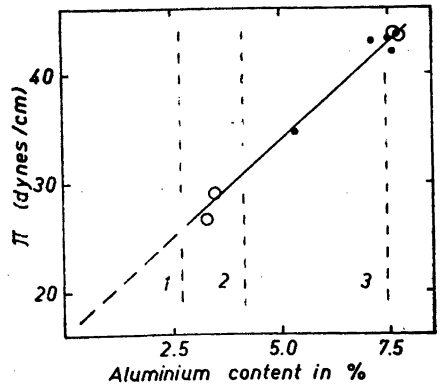

Fig. 4. The relation between the collapse pressure π_K and the aluminium content of the monolayer on substrates $1 \cdot 10^{-3}$ M in $KAl(SO_4)_2$. 20° C. The dotted lines give theoretical aluminium content for different aluminium rosinates. 1 aluminium trirosinate, 2 monobasic aluminium dirosinate, 3 dibasic aluminium monorosinate.

where the properties of a typical aluminium rosinate curve are fully developed and the aluminium content of the monolayer substance is that of the dibasic aluminium monorosinate becomes clearly evident. Up to this pH value, both curves run continuously.

Fig. 4 shows directly the variations of the collapse pressure of the monolayer with its aluminium content. There are no break points in the curve up to the aluminium content of the dibasic aluminium monorosinate. The conclusion thus seems justified that the reaction between aluminium ions and the rosin acid monolayer takes place without intermediate stages until the formation of aluminium monorosinate is complete and that the latter compound is the final product of the primary interaction. Before this end-point is reached, the monolayer is evidently a mixture of unreacted rosin acid and aluminium monorosinate.

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Kinetics of the Autoxidation of Cuprous Chloride in Hydrochloric Acid Solution

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It is shown that the velocity of the autoxidation of cuprous ion in hydrochloric acid solution is proportional to the concentrations of oxygen and cuprous ion. The velocity increases with increasing hydrogen ion concentration, the reciprocal velocity being a linear function of the reciprocal hydrogen ion concentration, provided $cH^+ \geq 0.1$. As the rate of reaction was high, it proved impossible to saturate the reacting solutions with oxygen, so that the oxygen concentration had to be determined by calculations assuming a steady state.

The kinetic expression found suggests the following mechanism:

$(O_2)_{gas}$	$\rightleftharpoons$	$(O_2)_{liquid}$	(± 1)
$O_2 + Cu^+$	$\rightleftharpoons$	CuO_2^+	(± 2)
$CuO_2^+ + H^+$	$\rightarrow$	$Cu^{++} + HO_2$	$(+3)$

followed by a sequence of fast steps in which three more cuprous ions per oxygen molecule are oxidised. The values of the rate constants for the individual steps are discussed.

It is well known that copper ions play an important part as a catalyst in many oxidations. The mechanism of this behaviour is generally associated with the ability of copper to exist in two oxidation states in solution [1]. To solve the kinetic problems of such reactions adequately it is necessary to know the kinetics of the oxidation of cuprous ion. The present investigation is of preliminary character as various factors which influence the rate have been varied within rather narrow limits only. The results have been used in evaluation of the kinetics and mechanism of the copper catalysed autoxidation of ascorbic acid, which is treated in a following paper.

The autoxidation of cuprous chloride in mixed solution of sodium chloride and hydrogen chloride was investigated. The chloride concentration was kept throughout at 1.000 *M*. The hydrogen ion concentration varied from 0.400 to 0.020 *M* and the sodium ion concentration correspondingly from 0.600 to 0.980 *M*. The initial concentration of chlorocuprous ion was generally about 0.008 *M*. It has been shown in this laboratory[2] that cuprous ions in such solutions exist essentially as $CuCl_2^-$.

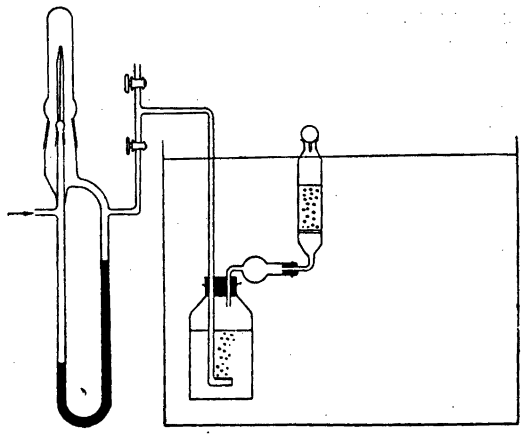

Fig. 1. Experimental set up (see text).

All reagents used were "Baker's analyzed". The cuprous chloride was washed with dilute hydrochloric acid to remove oxidised products, otherwise the chemicals were used as delivered. The solutions were made up under anaerobic conditions. 225 ml of suitable mixtures of 1.000 *M* hydrochloric acid and 1.000 *M* sodium chloride were deaerated in a two necked bottle by a stream of carbon dioxide. To this solution was added a saturated solution of cuprous chloride in 1 *M* sodium chloride, generally 25 ml.

As discussed below it was impossible to saturate the solutions with oxygen because of the high rate of the reaction. Therefore it was necessary to determine the progress of the reaction in separate runs each of which gave one interrelation between time and degree of reaction only. The reaction was carried out in a small reaction vessel (Fig. 1), a vertical glass cylinder with a sintered glass disc as a gas inlet in the bottom. The volume of the reaction vessel was about 25 ml.

In each run 15 ml of the reaction mixture was transferred from the stock solution in the two necked bottle by a pipette, the tip of which was somewhat enlarged to ensure quick delivery. The gas, a mixture of oxygen and nitrogen, generally with 4.05 % oxygen, was taken from a small steel cylinder and passed through a wash bottle with 1 *M* sodium chloride, submerged in the thermostat, to ensure proper temperature and humidity. The rate of the gas flow was carefully controlled by a flow meter. No temperature difference was detectable between the contents of the reaction vessel and the thermostat water.

At the end of the time scheduled for the individual runs the gas flow was discontinued by releasing the pressure below the glass disc. The amount of oxygen in the solution was generally so small compared with the cuprous ion concentration that no great error was introduced by further reaction. 10.00 ml of the reacted solution was transferred by means of a Krogh syringe to a sulphuric acid and ferric chloride solution, and the ferrous ion formed titrated with 0.01 *N* potassium permanganate with ferroin as an indicator. A small blank was subtracted.

Ferric salt was used as oxidant because cuprous ions do not react stoichiometrically with permanganate or with ceric sulphate [3]. When cuprous salt was titrated directly, *i.e.*, without addition of ferric ion, the results were found to depend on such things as acidity, rate of titration, and rate of carbon dioxide current. The method here described was tested on solid cuprous chloride purified in the following way: dissolution in concentrated hydrochloric acid, reprecipitation by dilution, washing with glacial acetic acid and drying at 130° C. The results were in agreement with stoichiometry within 0.5 %.

The first runs showed that the velocity of the reaction does not depend on illumination or on added cupric chloride. It was further shown that although hydrogen peroxide could be detected by the spot test with lead sulphide it was only formed in negligible amount and could not be determined by the usual quantitative methods. The stoichiometry of the reaction is therefore:

$$4\ Cu^{+} + 4\ H^{+} + O_2 \rightarrow 4\ Cu^{++} + 2\ H_2O$$

The reaction vessel described above ensures a very intimate contact between gas phase and solution. Even so it was impossible to approach a state of saturation with oxygen, so that the concentration of oxygen in the reacting solution had to be determined by a steady state method, *i.e.*, by calculations assuming the rate of flow of oxygen from the gas phase into the liquid to be equal to the flow of oxygen in the opposite direction plus the rate of oxygen consumption:

$$k_1\ pO_2 = k_{-1}cO_2 + s \qquad (I)$$

Here s is the experimentally determined speed of the reaction: $\left(-\frac{dcCu^{+}}{dt}\right)$, and k_1 and k_{-1} the first order constants of forward and backward reaction according to the scheme

$$(O_2)_{gas} \rightleftharpoons (O_2)_{liquid} \qquad (\pm 1)$$

The exact values of these constants are determined by the conditions of experiment but the ratio between them is of course the solubility of oxygen in the liquid according to Henry's law. Whenever s is small compared with the two other terms the oxygen concentration of the reacting solution will approach saturation. An early attempt to determine the kinetics of the autoxidation of cuprous ion was unsuccessful because s was very nearly equal to k_1pO_2, so that the oxygen transference apparently was the sole rate determining factor [4]. In the present investigation the upper limit for s was about semisaturation upwards. It is evident that a necessary condition for success was a careful control of the factors which determine the rate of diffusion of oxygen into the solution.

Fig. 2 demonstrates the proportionality between velocity and oxygen concentration in the liquid phase. We may write:

$$s = cO_2 f(c,h) \qquad (II)$$

where c is the cuprous ion concentration and h the hydrogen ion concentration. The elimination of cO_2 between I and II gives:

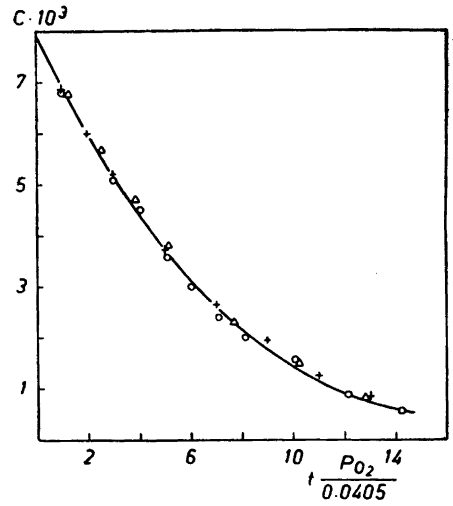

Fig. 2. Demonstration of the proportionality between rate and oxygen pressure in gas phase.

Ordinate: Cuprous ion concentration.

Abscissa: Time multiplied by oxygen pressure, divided by 0.0405. All times are hence referred to $pO_2 = 0.0405$.

△ ○ × denote values from experiments with [H+] = 0.100 and with oxygen pressures 0.208, 0.082, 0.0405

$$s = -\frac{dc}{dt} = \frac{k_1 pO_2 \cdot f(c,h)}{k_{-1} + f(c,h)}$$

or

$$\frac{1}{s} = -\frac{dt}{dc} = \frac{1}{k_1 \cdot pO_2} + \frac{k_1}{k_1 \cdot pO_2 \cdot f(c,h)} \qquad \text{(III)}$$

The integral form is:

$$t = \frac{c_o - c}{k_1 \cdot pO_2} + \frac{k_{-1}}{k_1 \cdot pO_2} \cdot \int_c^{c_o} \frac{dc}{f(c,h)} \qquad \text{(IV)}$$

The first term on the right hand side of (IV) can be determined in two ways, either by direct experiments or indirectly by analysis of the experimental material from the main experiments. The accuracy of this investigation being rather low, it was considered unfavourable to use the latter method. Inspection of (I) shows that k_1 may be easily determined if it is possible to design experiments in which the steady state concentration of oxygen in the liquid phase is nearly zero. Therefore s was determined in experiments with solutions of sodium sulphite or with higher concentrations of cuprous chloride. Using sulphite it was found that the velocity of reaction was independent of sulphite

concentration and proportional to the oxygen pressure of the gas phase. For the experimental conditions used in the investigation was found:

$$s = 0.0483 pO_2 \text{ equivalent/liter} \cdot \text{minute}$$

The initial velocity of oxidation of concentrated solutions of cuprous chloride was nearly zero order in cuprous ion and proportional to the oxygen pressure. This velocity was almost identical with the velocity from the sodium sulphite experiments, so no doubt these velocities are actually the rate at which oxygen is transferred from gas to liquid.

Knowing k_1 we can determine the first term on the right hand side of (IV) and define a new parameter:

$$t' = t - \frac{c_o - c}{k_1 \cdot pO_2} = \frac{k_{-1}}{k_1 \cdot pO_2} \int_c^{c_o} \frac{dc}{f(c,h)} \qquad (V)$$

Here t' is the time the reaction from c_o to c would have taken had the solution been saturated with oxygen. The influence of the oxygen deficiency is thus eliminated.

It was now found that ln c is nearly a linear function of t'. This indicates linearity between velocity and c. Strict linearity of the logarithmic plot cannot be expected as the velocity increases with acidity, which again falls as the reaction proceeds. The relation between reciprocal velocity and reciprocal hydrogen ion concentration is nearly linear when $h \geq 0.1$. In less acid solution the rate is higher than corresponding to this relationship. We shall confine the attention to the acidity region in which the simple relation holds.

Analysis of the available experimental material gives the expression

$$\frac{1}{s} = -\frac{dt}{dc} = \frac{20.7}{pO_2} + \frac{1.62 \cdot 10^{-2}}{pO_2 \cdot c} + \frac{1.40 \cdot 10^{-2}}{pO_2 \cdot c \cdot h} \qquad (VI)$$

Introducing $h = h_o - c_o + c$, h_o and c_o being initial values of h and c, the integration gives:

$$t = \frac{(c_o - c) \cdot 20.7}{pO_2} + \frac{1.62 \cdot 10^{-2}}{pO_2} \cdot \ln \frac{c_o}{c} + \frac{1.40 \cdot 10^{-2}}{pO_2 (h_o - c_o)} \ln \frac{(h_o - c_o + c) \cdot c_o}{h_o \cdot c} \qquad (VII)$$

The acidity change during the experiments was so small that the following approximation is useful

$$\ln \frac{h_o - c_o + c}{h_o} = -\frac{c_o - c}{h_o}$$

Therefore

$$t = \left[\frac{20.7}{pO_2} + \frac{1.40 \cdot 10^{-2}}{pO_2 \cdot (h_o - c_o) h_o}\right](c_o - c) + \left[\frac{1.62 \cdot 10^{-2}}{pO_2} + \frac{1.40 \cdot 10^{-2}}{pO_2 \cdot (h_o - c_o)}\right] \ln \frac{c_o}{c} \qquad (VIII)$$

Table 1. I and II mean zero- and first order terms respectively. pO_2 was 0.208, initial hydrogen ion concentration 0.100. Hence the zero order factor was 92.5 and the first order factor 0.808.

t obs.	$c \cdot 10^3$	I	$\ln c \cdot 10^3$	II	t calc.
0	8.01	0	2.081	0	0
0.25	6.78	0.11	1.914	0.13	0.24
0.50	5.65	0.22	1.732	0.20	0.42
0.75	4.70	0.30	1.548	0.47	0.77
1.00	3.80	0.39	1.335	0.66	1.05
1.50	2.30	0.53	0.833	1.01	1.54
2.00	1.51	0.60	0.412	1.35	1.95
2.50	0.82	0.66	−0.128	1.96	2.62
3.00	0.43	0.71	−0.844	2.36	3.07
3.50	0.24	0.73	−1.427	2.83	3.56

Table 2. pO_2 was 0.0405, initial hydrogen ion concentration 0.400. Hence the zero order factor was 512 and the first order factor 1.28.

t obs.	$c \cdot 10^3$	I	$\ln c \cdot 10^3$	II	t calc.
0	7.80	0	2.054	0	0
0.5	7.05	0.38	1.953	0.13	0.51
1.0	6.32	0.76	1.844	0.27	1.03
1.5	5.67	1.09	1.735	0.41	1.50
2.0	4.95	1.46	1.599	0.58	2.04
3.0	3.70	2.10	1.308	0.95	3.05
4.0	2.60	2.66	0.956	1.41	4.07
5.0	1.74	3.10	0.554	1.92	5.02
6.0	1.14	3.38	0.131	2.46	5.84

The agreement between (VIII) and experiment is demonstrated in the Tables 1 and 2. The coefficients of the two terms are calculated from the experimental conditions. The expression is then used to calculate t, and the agreement between theory and experiment is illustrated by the concordance between experimental and calculated values of t.

The experimental expression, *e.g.* equation (VI), suggests the following mechanism:

$$(O_2)_{gas} \rightleftharpoons (O_2)_{solution} \qquad (\pm 1)$$

$$Cu^+ + O_2 \rightleftharpoons CuO_2^+ \qquad (\pm 2)$$

$$CuO_2^+ + H^+ \rightarrow Cu^{++} + HO_2 \qquad (\pm 3)\ *$$

The corresponding expression for the reciprocal rate, as derived by a steady treatment, is [5]:

$$\frac{1}{s} = \frac{1}{k_1 pO_2} + \frac{k_{-1}}{k_1 k_2 \cdot pO_2 \cdot c} + \frac{k_{-1}k_{-2}}{k_1 k_2 k_3 pO_2 \cdot c \cdot h} \qquad (IX)$$

* Strictly speaking nothing is known about the products of reaction (+ 3).

which is identical with (VI). The third step must be irreversible, otherwise the rate would depend on the cupric ion concentration. Almost certainly three more cuprous ions are oxidised by the HO_2 radical in a sequence of fast reactions, but these steps are not kinetically perceptible as the terms after the third must be small when the rate is uninfluenced by cupric ion concentration. The only alternative to this mechanism is reformation of oxygen by reactions between the partly reduced oxygen molecules (HO_2, H_2O_2, HO), which is clearly improbable because such molecules would be rare.

It should now be emphasized that this expression is valid only when $cH^+ \geq 0.1$. At lower hydrogen ion concentrations the rate is somewhat higher than corresponding to the expression. The reason for this complex relationship is probably that the cuprous-oxygen complex formed in the second step may react further in two ways, one of which is reaction (+ 3) in the mechanism given above. It may be assumed that (+ 3) has a dominating probability compared with an alternative mechanism (which does not involve hydrogen ions) when hydrogen ion concentration is 0.1 or greater, but that the other mechanism gains significance when the hydrogen ion concentration is essentially lower. The deviation between (VII) and experiment is about 15 % at $cH^+ = 0.05$ and about 40 % at $cH^+ = 0.02$. Universal agreement would imply that cuprous ions are not oxidised in neutral solution.

We shall now investigate what information we can get from the absolute values of the constants. Comparison of (VI) and (IX) gives:

$$k_1 = 0.048; \qquad \frac{k_1 k_2}{k_{-1}'} = 62; \qquad \frac{k_1 k_2 k_3}{k_{-1} k_{-2}} = 71$$

The solubility of oxygen in water at 25° C is about 10^3 mole/atmosphere, so $k_{-1}k_{-1} = 10^{-3}$. Therefore k_2 is about $60 \cdot 10^3$ $mole^{-1} \cdot minute^{-1}$, or 10^3 $mole^{-1} \cdot sec^{-1}$. The kinetic expressions derived above do not take into account that presumably four cuprous ions are oxidised every time one passes through the rate determining steps, so k_2 is actually about 250. The Arrhenius equation

$$k = Z \cdot \exp(-E/RT) \qquad (X)$$

where Z is the frequency factor and E the energy of activation would with "normal" Z value, 10^{11}, suggest an energy of activation of roughly 12 000 calories per mole.

k_{-2}/k_3 is nearly one. This means that a CuO_2^+ complex when formed has about equal prabability to react according to (+3) and (—2) in a solution which is 1 M in hydrogen ions. Christiansen [6] has pointed out that at least one of the two reactions in an element of a sequence of reactions has zero energy of activation. As now both (+ 2) and (— 3) are slow we would expect (—2) and (+ 3) to be fast reactions where the exponential factor of (X) are unity. The closeness to unity of k_{-2}/k_{+3} indicates that this condition is fulfilled, although the value should rather have been 10^2, as the most probable value (according to the mechanism suggested) of the frequency factor would be 10^{13} for k_{-2}, corresponding to a monomolecular reaction, and 10^{11} for k_3, as (+ 3) is bimolecular. The value 1 might suggest that (—2) actually is bimolecular, *e.g.* a reaction between CuO_2^+ and chloride ion, which was present at molar con-

centration in all experiments. As a matter of fact it was found that the reaction proceeds much faster when the chloride concentration was reduced.

A result of this investigation, the indication of the existence of a complex between cuprous ion and oxygen, is an extention of the analogy between cuprous ion and haemoglobine, shown by their similar behaviour towards carbon monoxide.

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Applicability of the Steady State Principle to the Autoxidation of Cuprous Chloride

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The *steady state equation* for determination of oxygen in a solution through which an oxygen containing gasphase is blown, and in which oxygen is being consumed by a chemical reaction, is compared with the *differential equation* for the oxygen concentration. The error introduced by determination of the oxygen concentration by using the simpler equation is calculated, and is negligible.

It is customary in chemical kinetics to base the treatment of the overall rate problem for a sequence of reactions on the assumption that the system will quickly settle down to a state in which the rates of appearance and disappearance of molecular species, which do not occur in the stoichiometric reaction scheme, are equal. This method is only strictly valid for systems which are invariant in time, but the difference for other systems between treatments based on this principle and rigorous solutions of the system of simultaneous differential equations for all species in the given problem is very often far below the limits of experimental accuracy. The steady state principle is always applicable in cases where the intermediates are so shortlived that they only represent a negligible amount of the reaction system in comparison with the concentrations of the stoichiometric components. Sometimes it is, however, convenient to apply steady state treatment to systems where this condition is not strictly fulfilled, and in such cases it is desirable to have a method to test the reliability of the method.

A problem of this kind is found in the preceding paper[1]. There it was found that the rate with which oxygen was used in a certain reaction was so high that the oxygen concentration in the reacting solution was essentially below saturation when a mixture of oxygen and nitrogen was blown through the reaction vessel. The oxygen concentration was in this case determined by the steady state principle, *i.e.*, by the equation

$$k_1 pO_2 - k_{-1} cO_2 - s = 0$$

(equation (I) in the preceding paper)

where everything except cO_2 was known. The problem should strictly have been solved by integration of the differential equation

$$\frac{dcO_2}{dt} = k_1 pO_2 - k_{-1} cO_2 - s$$

with appropiate boundary conditions. The objections against the use of the steady state principle in kinetics originate in the difference between these two equations, the apparent paradox introduced by equating to zero the rate of change of something which actually does change.

In the present problem the first term on the right hand side of the differential equation is a constant, and the next two terms are proportional to the oxygen concentration, s being a function of time. The problem is now to determine the difference between a determined by the equation

$$a - a(b + y) = 0 \qquad \text{(I)}$$

and by integration of the differential equation

$$\frac{da}{dt} = a - a(b + y) \qquad \text{(II)}$$

the boundary condition being that $a = 0$ for $t = 0$.

The general solution of the differential equation is

$$a = a \cdot e^{-\int_0^t (b+y)\,dt} \int_0^t e^{\int_0^\tau (b+y)\,d\tau} dt \qquad \text{(III)}$$

This expression can be transformed in a convenient way by means of the following relations:

$$\int e^{F(x)} dx = \int \frac{1}{f(x)} \cdot e^{F(x)} dF(x) = \frac{1}{f(x)} \cdot e^{F(x)} - \int \frac{d1/f(x)}{dF(x)} e^{F(x)} dF(x) =$$

$$= \frac{1}{f(x)} e^{F(x)} - \int \frac{d1/f(x)}{dx} e^{F(x)} dx = \frac{1}{f(x)} e^{F(x)} + \int \frac{f'(x)}{f(x)^2} e^{F(x)} dx;$$

$$f(x) \equiv F'(x)$$

The boundary condition given above is satisfied when the integration constant

$$\frac{1}{f(0)}$$

is subtracted. Introduction into the general solution for the differential equation gives:

$$a = a \cdot e^{-\int_0^t (a+y)dt} \left\{ \frac{1}{b+y} e^{\int_0^t (a+y)dt} - \frac{1}{b+y_0} - \int_0^t \frac{y}{(b+y)^2} e^{\int_0^\tau (b+y)\,d\tau} dt \right\}$$

$$a = \frac{a}{b+y} - \frac{a}{b+y_0} e^{\int_0^t (a+y)dt} - a\, e^{-\int_0^t (a+y)dt} \int_0^t \frac{y'}{(b+y)^2} e^{\int_0^\tau (b+y)d\tau} dt \qquad \text{(IV)}$$

This expression is identical with the general solution above. The first term is identical with the solution of the steady state equation (I). The second term is the initial value of the steady state solution multiplied by an exponential factor. It is easy to investigate how quickly this term vanishes in a given problem. The third term is identical with (III) apart from the factor immediately after the big sign of integration. The contribution from this term is determined by calculation of this factor, and is negligible if

$$\frac{y'}{(b+y)^2} << 1.$$

In the preceding paper it was found that k_1 for the applied experimental conditions has the value

0.0483 equivalent/liter · minute · atmosphere

In the state of saturation we have:

or $$a = b \cdot \alpha$$

$$k_1 pO_2 = k_{-1} \cdot cO_2$$

As now $$\frac{pO}{cO} \sim 800 \text{ atmospheres/mole}$$

we have $$k_{-1} = 60$$

y is the function, which by multiplication with cO_2 (α) gives s, so we have

$$y \cdot cO_2 = \frac{62.6 \cdot c \cdot h}{0.875 + h} \cdot pO_2 = \frac{50\,000 \cdot c \cdot h}{0.875 + h} \cdot cO_2$$

Introduction of $c = 0.01$ and $h = 0.1$ gives

$$y = 50$$

so the exponential factor has the value

$$e^{-110t}$$

which reaches the value 0.01 in less than three seconds.

Under the chosen experimental conditions we have:

$$y \sim 5\,000 \cdot c$$

or

$$y' \sim 5\,000 \cdot \frac{dc}{dt}$$

We were (preceding paper equation (VI)) able to express the variation of c in time by the empirical formula

$$-\frac{dt}{dc} = \frac{20.7}{pO_2} + \frac{1.62 \cdot 10^{-2}}{pO_2 \cdot c} + \frac{1.40 \cdot 10^{-2}}{pO_2 \cdot c \cdot h}$$

Introduction of the values for c and h above and $pO_2 = 0.2$ gives

$$-\frac{dt}{dc} \sim 100 + 30 + 300 = 430$$

or

$$-\frac{dc}{dt} \sim 0.002$$

This gives $y' = -5\,000 \cdot 0.002 = -10$
We therefore have

$$-\frac{y'}{(k_{-1} + y)^2} \sim \frac{10}{110^2} \sim 0.0008 << 1$$

Thus also the third term of (IV) is small and it is seen that the special application of the steady state principle in the preceding paper is fully justified.

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Kinetics and Mechanism of the Copper Catalysed Autoxidation of Ascorbic Acid

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Based on a discussion of earlier investigations of the copper catalysed reaction between oxygen and ascorbic acid the following mechanism is suggested

$$\begin{aligned} H_2A &\rightleftharpoons HA^- + H^+ \\ HA^- + Cu^{++} &\rightleftharpoons HA + Cu^+ \\ HA &\rightleftharpoons A^- + H^+ \\ A^- + Cu^{++} &\rightarrow A + Cu^+ \\ 2\,Cu^+ + 2\,H^+ + \tfrac{1}{2}O_2 &\rightarrow 2\,Cu^{++} + H_2O \\ \hline H_2A + \tfrac{1}{2}O_2 &\rightarrow A + H_2O \end{aligned}$$

The corresponding kinetic expression is derived, certain justified approximations being introduced. It is shown that there is complete agreement between the integrated velocity expression and the course of the reaction as determined by experiment. From the kinetic expression the steady state distribution of copper in the two oxidation states is calculated for the conditions of a special experiment, and the values calculated are compared with the result of a spectrophotometric determination of cupric ion as a function of time in an identical experiment. The values of the velocity constants for the single steps are derived from the experiments, and a discussion proves them to be reasonable. It is therefore evident that the above mechanism is correct.

The most remarkable feature in the chemistry of ascorbic acid is its reaction with oxygen, strongly and specifically catalysed by copper ions. In view of the physiological importance of ascorbic acid it is understandable that most of the available investigations of the kinetics of this reaction have been carried out in solution near the physiological pH region. However, the protolytic properties of ascorbic acid, and the ability of copper ions to form complexes with various components of buffered solutions, introduce unnecessary complications, the result being that none of the earlier investigators have been able to provide experimental material from which an established mechanism can be derived. In the present investigation an attempt is made to overcome these difficulties by a choice of conditions much more convenient from the point of

view of physical chemistry. In this way it proves possible to remove the uncertainty completely, and thence to derive a mechanism.

In previous investigations it is almost universally maintained that the autoxidation of ascorbic acid in the presence of copper ion is a transference catalysis:

$$\begin{array}{rcl} H_2A + 2Cu^{++} & \rightarrow & A + 2Cu^{+} + 2H^{+} \\ 2Cu^{+} + 2H^{+} + O_2 & \rightarrow & 2Cu + H_2O \\ \hline H_2A + O_2 & \rightarrow & A + H_2O \end{array}$$

(H_2A = ascorbic acid; A = dehydro ascorbic acid).

The most careful investigations of the kinetics of this reaction are due to Barron, de Meio and Klemperer [1], Weissberger and LuValle [2] and Lyman *et al.*[3] Barron *et al.*[1] found in 1936 that the rate of the uncatalysed reaction is negligible at pH < 7.6. In alkaline solution ascorbic acid reacts with one molecule of oxygen, even without a catalyst, forming oxalic acid and L-treonic acid. This reaction is, as opposed to the catalytic reaction in neutral or acid solution, irreversible and uninhibited by cyanide ion. At pH values lower than 6.6, in the presence of copper, one molecule of ascorbic acid reacts with one atom of oxygen only, forming dehydro ascorbic acid as by the oxidations with iodine or 2,6-dichlorophenoleindophenole. Accordingly the ascorbic acid is recovered quantitatively by reduction with hydrogen sulphide. The velocity of the reaction, according to Barron *et al.*, increases with increasing copper concentration, but less than corresponding to a simple proportionality law. The rate decreases with increasing acidity, but is still measurable in 0.1 *M* hydrochloric acid. The rate increases with oxygen concentration, but less than according to proportionality. These are the facts on which Barron *et al.* based the undetailed transference mechanism. They are all confirmed by the present investigation.

Weissenberger and LuValle [2] suggest that the rate determining step in the copper catalysed autoxidation of ascorbic acid is

$$HA^{-} + Cu^{++} \rightarrow HA + Cu^{+}$$

the semiquinone (HA) and the cuprous ion formed both reacting further with oxygen. All steps except this one are considered fast. This is hardly in accordance with an experimental finding by the same authors: that the velocity is proportional to the oxygen concentration. The complex relationship between velocity and copper concentration is, according to Weissberger and LuValle, associated with the ability of copper to form complexes with buffer components.

Ball [4] has determined the redox potential of the reaction

$$H_2A \rightleftharpoons A + 2\ H^{+} + 2\ e^{-}$$

to be 0.390 volt. This potential has been verified by various investigators. Fenwick [5] has determined the potential for the reaction

$$Cu^{+} \rightleftharpoons Cu^{++} + e$$

to be 0.167 volt. These figures mean that the equilibrium

$$H_2A + 2\ Cu^{++} \rightleftharpoons A + 2\ Cu^{+} + 2\ H^{+}$$

in acid solution is displaced to the left hand side. The solutions used in the present investigation was 1.000 *M* in chloride ion throughout. Therefore the formation of dichloro cuprous ion was significant for the distribution of copper on the two oxidation states:

$$H_2A + 2\ Cu^{++} + 4\ Cl \rightleftharpoons A + 2\ CuCl_2^- + 2\ H^+$$

The dissociation constant of the dichloro cuprous ion is estimated by Latimer [6] as 2.9×10^{-6} and by Bjerrum [7] as about 3.0×10^{-6}. These values combined with the redox potential for the free cuprous ion gives about 0.487 volt for the reaction

$$CuCl_2 \rightleftharpoons Cu^{++} + 2\ Cl^- + e^-$$

Handbook of Physics and Chemistry (1947) p. 1418, quotes 0.455 volt. The writer has been unable to trace the origin of this value, but has confirmed it by his own measurements. Cupric ion is thus almost completely reduced by ascorbic acid in acid chloride solution.

Lyman *et al.*[3] have shown that the velocity of the copper catalysed autoxidation in 0.1 *N* sulphuric acid solution is vanishingly small, but quite measurable in 0.1 *M* hydrochloric acid solution, where the rate as a function of the hydrochloric acid concentration has a minimum. In the present investigation it was found that the velocity of the reaction is zero in 0.1 *M* perchloric acid and conveniently measurable in a solution which is 0.1 *M* in hydrochloric acid and 0.9 *M* in sodium chloride.

The first two steps in the mechanism suggested by Weissberger and LuValle were

$$H_2A \rightarrow HA^- + H^+ \qquad (+1)$$

$$HA^- + Cu^{++} \rightarrow HA + Cu^+ \qquad (+2)$$

These steps are essentially uninfluenced by chloride ions, so apparently they are not sole rate-determining. Chloride ion will trap the cuprous ion formed by (+ 2) and therefore prevent or at least grossly inhibit the reaction

$$HA + Cu^+ \rightarrow HA^- + Cu^{++} \qquad (-2)$$

The third step in the Weissberger and LuValle mechanism is

$$HA \rightarrow H^+ + A^- \qquad (+3)$$

which we shall accept. The fourth step suggested is

$$A^- + O_2 \rightarrow A + O_2^-$$

which seems very unlikely as the concentrations of copper and oxygen are comparable. There is no obvious reason to assume that A^- should prefer to react with oxygen when HA^- prefers the cupric ion. The fourth step is rather

$$A^- + Cu^{++} \rightleftharpoons A + Cu^+ \qquad (\pm 4)$$

In a preceding paper it was suggested that the oxygen molecule is reduced stepwise by four cuprous ions, the final product being water. Therefore a solution in which oxidation of cuprous ion is going on must contain partly reduced oxygen molecules such as H_2O, H_2O_2 and HO. Experiments in which cuprous ion was oxidised in the presence of oxalic acid, formic acid or pyrogallol showed that these organic compounds are not subject to "induced oxidation",

the organic compounds being present quantitatively after the experiments, and the rate of oxidation of cuprous ion being unimpaired by their presence. It is therefore suggested that the following reactions are insignificant:

$$H_2A + HO_2 \rightarrow ?$$
$$H_2A + H_2O_2 \rightarrow ?$$
$$H_2A + HO \rightarrow ?$$

We shall also neglect

$$HA^- + HO_2 \rightarrow ?$$
$$HA + HO_2 \rightarrow ?$$
$$A^- + HO_2 \rightarrow ?$$

etc. as the ascorbic acid derivatives are rare in the reacting solutions compared with the cuprous ions with which the oxygen derivates are supposed to react fast. Also the possibility that the complexes $CuHA^+$ and CuA^+ have a significant lifetime is disregarded. It is well known that copper complexes with polyalcohols and similar compounds do not exist in acid solution.

As a result of the introductory discussion, the following mechanism is suggested:

$$H_2A \rightleftharpoons H^+ + HA^- \qquad (\pm 1)$$
$$HA^- + Cu^{++} \rightleftharpoons HA + Cu^+ \qquad (\pm 2)$$
$$HA \rightleftharpoons A^- + H^+ \qquad (\pm 3)$$
$$A^- + Cu^{++} \rightarrow A + Cu^+ \qquad (+ 4)$$
$$2\ Cu^+ + 2\ H^+ + \tfrac{1}{2}O_2 \rightarrow 2\ Cu^{++} + H_2O \qquad (5)$$
$$H_2A + \tfrac{1}{2}O_2 \rightarrow A + H_2O$$

The overall velocity of this reaction will be a function of the concentrations of ascorbic acid, hydrogen ion, copper ion and oxygen. It is also influenced by temperature and chloride ion concentration, but these two factors were kept constant, their variation not being necessary to solve the problem set, to establish a mechanism of the reaction. In the individual runs all factors except the ascorbic acid concentration (and of course the concentration of dehydro ascorbic acid) were constant. It is our task to calculate what kind of function of time the ascorbic acid concentration should be in an experiment were the above mechanism right. The method used is that of steady state calculations, *i.e.*, writing down of equations assuming the rates of appearance and disappearance of the intermediate molecular species to be equal. From these equations the concentrations of the intermediates are then eliminated.

The system of equations would have been linear in the ascorbic acid concentration, c, had each step involved formation and disappearance of not more than one intermediate. Whenever two intermediates appear on the same side of an element of the sequence the degree of the kinetic expression is raised by one. As both the ascorbic acid derivatives (except dehydro ascorbic acid) and the two oxidation steps of copper are intermediates in the reaction-kinetic sense of the word, this occurs three times: in the reactions (+ 2), (— 2) and (+ 4). Therefore our expression will be strictly one of the fourth degree in c. Obviously certain approximations are necessary to solve the system of equations.

One simplification is justified by the following: When an ascorbic acid ion (HA^-) is formed it may either react with a cupric ion, (+2), or it may recombine with a hydrogen ion, (—1). Hydrogen ions are much more abundant than cupric ions, so the probability of (—1) is therefore likely to be much higher than that of (+2). The final result of the present investigation is that only few of the encounters between cupric ion and ascorbic acid ion lead to reaction according to (+2), so (—1) is definitely the fate of the great majority of ascorbic acid ions. Therefore the concentration of ascorbic acid ion (HA^-) can be calculated simply by using the mass action law on the dissociation according to (1). This simplication reduces the degree of our system of equations to three.

A great simplification is introduced by the experimental finding that the velocity of the reaction is uninfluenced by the concentration of dehydro ascorbic acid, *i. e.*, the velocity of (—4) is almost zero. This is only true when the system investigated contains chloride ions to trap the cuprous ions formed by (+2) and by (+4). Otherwise (—4) is so dominating that the overall rate of the reaction is negligible.

It is an important assumption that the state of the cuprous chloride system is the same as in the solutions used in a previous investigation [8] of the cuprous ion autoxidation.

We are now able to write down the equations necessary to derive the overall kinetics according to the mechanism suggested:

$$0 = [\dot{HA}] = k_2[HA^-][Cu^{++}] - k_{-2}[HA][Cu^+] - k_3[HA] + k_{-3}[A^-][H^+] \quad (I)$$

$$0 = [\dot{A}^-] = k_3[HA] - k_{-3}[A^-][H^+] - k_4[A^-][Cu^{++}] \quad (II)$$

$$0 = [\dot{Cu}^+] = -[\dot{Cu}^{++}] = k_2[HA^-][Cu^{++}] - k_{-2}[HA][Cu^+] + k_4[A^-][Cu] - 2s \quad (III)$$

where the dots denote differentiation with respect to time.

s is the rate of disappearance of ascorbic acid, $-\frac{dc}{dt}$, and two cuprous ions are oxidised in each cycle of the reaction. We thus have three equations with five unknowns. The distribution of copper on two oxidation states gives a fourth equation:

$$[Cu^+] + [Cu^{++}] = (Cu) \quad (IV)$$

where complex formation between copper and chloride is disregarded, and (Cu) is the total copper concentration. The remaining equation is

$$s = f([Cu^+]\, pO_2\, [H^+]) \quad (V)$$

as found in the preceding investigation [8].

Elimination of [HA] and [A^-] from the first three equations gives:

$$s = \frac{k_2k_3k_4[HA^-][Cu^{++}]^2}{k_3k_4[Cu^{++}] + k_{-2}k_4[Cu^+][Cu^{++}] + k_{-2}k_{-3}[Cu^+][H^+]}$$

$$\text{or } \frac{1}{s} = \frac{1}{k_2[HA^-][Cu^{++}]} + \frac{k_{-2}[Cu^+]}{k_2k_3[HA^-][Cu^{++}]} + \frac{k_{-2}k_{-3}[H^+][Cu^+]}{k_2k_3k_4[HA^-][Cu^{++}]^2} \quad (VI)$$

In the preceding paper it was found that the velocity of the oxidation of cuprous ion at 25° C in a medium which is 1.000 *M* in chloride ion and at least 0.1 *M* in hydrogen ion is

$$s' = \frac{61.8 \cdot pO_2 \cdot [Cu^+][H^+]}{0.863 + [H^+]}$$

where pO_2 is the partial pressure of oxygen in a gas phase with which the solution is in equilibrium. The velocity with which ascorbic acid is oxidised is, according to the mechanism suggested, equal to the velocity of oxidation of two cuprous ions:

$$s = \frac{30.9\ pO_2[Cu^+][H^+]}{0.863 + [H^+]} = H[Cu^+] \qquad \text{(VII)}$$

where H is a known function of experimental conditions.

Introduction of K_s, the acid dissociation constant of ascorbic acid, gives

$$[HA^-] = \frac{[H_2A]}{[H^+]} K_s = \frac{c}{[H^+]} K_s$$

We further define:

$$[Cu^+] = \alpha\,(Cu) \text{ and } [Cu^{++}] = (1-\alpha)\,(Cu) \qquad \text{(VIII)}$$

We can therefore rewrite (VI):

$$\frac{1}{s} = \frac{1}{H\alpha\,(Cu)} \frac{[H^+]}{K_s k_2 \cdot c(1-\alpha)(Cu)} + \frac{k_{-2}[H^+]\alpha\,(Cu)}{K_s k_2 k_3\, c\,(1-\alpha)(Cu)} + \frac{k_{-2}k_{-3}[H^+]^2\alpha\,(Cu)}{K_s k_2 k_3 k_4\, c\,(1-\alpha)^2\,(Cu)^2}$$

Introducing simpler symbols for constants and concentrations not changed in individual experiments gives:

$$\frac{1}{\alpha} = \frac{H}{L\,c\,(1-\alpha)} \left\{ 1 + \frac{(Cu)}{M}\alpha + \frac{1}{N}\frac{\alpha}{1-\alpha} \right\} \qquad \text{(IX)}$$

where $L = \frac{K_s k_2}{[H^+]}, M = \frac{k_3}{k_{-2}}$ and $N = \frac{k_3 k_4}{k_{-2}k_{-3}[H^+]}$.

(IX) is an equation between c and α. (VII) and (VIII) define a simple relationship between s and α. From this is seen that were the second term in the bracket of equation (IX) vanishingly small compared with the other terms. the velocity of the reaction would be proportional to (Cu). Without this term (IX) is a second degree equation. As it turns out that the velocity of the reaction is nearly, but not quite, proportional to (Cu) it proves useful to solve (IX) as a quadratic equation, *i. e.*, without considering the variation of the term in question:

$$\frac{Lc}{H}\left(\frac{1-\alpha}{\alpha}\right)^2 - \frac{1-\alpha}{\alpha} - \left\{ \frac{(Cu)\,(1-\alpha)}{M} + \frac{1}{N} \right\} = 0$$

$$\frac{1-\alpha}{\alpha} = \frac{1 + \sqrt{1 + \frac{4Lc}{H}\left(\frac{(Cu)\,(1-\alpha)}{M} + \frac{1}{N}\right)}}{2\frac{Lc}{H}}$$

$$\frac{1}{\alpha} = 1 + \frac{H}{2Lc}(1 + \sqrt{1 + Rc}) \qquad \text{(X)}$$

where $$R = \frac{4L}{H}\left(\frac{(Cu)\ (1-\alpha)}{M} + \frac{1}{N}\right) \qquad (XI)$$

Introduction of $\frac{1}{s} = -\frac{dt}{dc} = \frac{1}{H\alpha\,(Cu)}$ gives

$$\frac{1}{s} = \frac{1}{H(Cu)} + \frac{1}{2L\,(Cu)}\left(\frac{1}{c} + \frac{1}{c}\sqrt{1+Rc}\right)$$

or

$$t = \int_{c}^{c_o}\left[\frac{1}{H\,(Cu)} + \frac{1}{2L\,(Cu)}\left(\frac{1}{c} + \frac{1}{c}\sqrt{1+Rc}\right)\right] dc$$

c_o being the initial concentration of ascorbic acid. Considering R constant we have

$$t = \frac{c_o - c}{H\cdot\,(Cu)} + \frac{1}{2L\,(Cu)}\left[\ln c + 2\sqrt{1+Rc} - 2\,\coth^{-1}\sqrt{1+Rc}\right]_{c}^{c_o}$$

which is not changed when $\ln x = \ln Rc$ is substituted for $\ln c$, so we have:

$$t = \frac{c_o - c}{H\,(Cu)} + \frac{1}{2L\,(Cu)}\left[\ln x + 2\sqrt{1+x} - 2\,\coth^{-1}\sqrt{1+x}\right]_{c}^{c_o} \qquad (XII)$$

As H is known from the preceding investigation we can calculate the zero order term, and so define:

$$t' = t - \frac{c_o - c}{H\,(Cu)}$$

The ascorbic acid used in the present investigation was a synthetic product from Hoffmann-La Roche. The experimental set up was the same as in the previous paper. As the absolute rate of the reaction is low compared with the rate of dissolution of oxygen, the reacting solution was nearly saturated with oxygen at the partial pressure of the oxygen-nitrogen mixture blown through the solution. The course of the reaction was followed by extraction of 1.000 ml samples from the solutions in the reaction vessel at suitable intervals by means of a Krogh syringe. The samples were transferred to a small titration vessel in which they were diluted to about 4 ml by the washing of the syringe with oxygenfree water, and they were stirred and de-oxygenated by a fast stream of carbon dioxide during the titration. The titrations were performed with 0.1 N I^-_3 solution with starch as an indicator. The burette was a 100 μl calibrated Rehberg micro burette.

The nature of the course of the reaction is shown on Fig. 1. It is seen that "the order of the reaction" is between zero and one. It is further seen that the velocity falls off as the acidity is increased. The velocity increases with oxygen concentration, but less than according to a proportionality law. The velocity is most sensitive to change of oxygen concentration at the lower acidity.

In chemical kinetics it is usual to investigate a first order reaction by plotting $\ln c$ against t, the proof of the first order character being that the plot is a straight line. In the present case (see XII) a plot of the function

$$y = \ln x + 2\sqrt{1+x} - 2\,\coth^{-1}\sqrt{1+x}$$

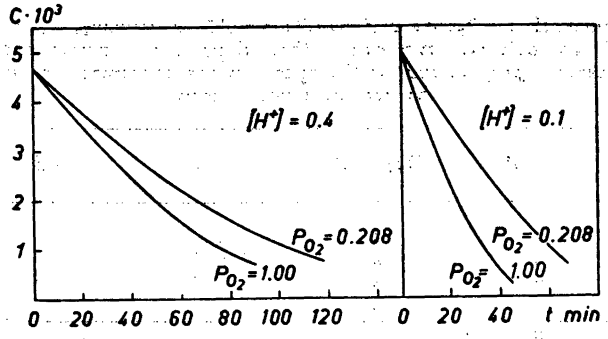

Fig. 1. Ordinate: Concentration of ascorbic acid. Abscissa: Time. It is seen that the rate is higher at the lower hydrogen ion concentration. Further that the rate is more sensitive to change of oxygen concentration at the lower hydrogen ion concentration.

against t' should be linear. Furthermore, when the conditions of experiment are changed, the variation of the reciprocal slopes from plots corresponding to individual experiments should vary in agreement with the coefficient 1/2L(Cu), *i. e.*, the slopes should be proportional to the concentration of copper and to the reciprocal hydrogen ion concentration, but should be independent of the oxygen concentration. The problem is therefore to investigate whether it is possible to assign such values to R, defined by (XI), that these conditions are fulfilled. We reintroduce the original meanings of the symbols L, M and N into R and have:

$$\ln x = \ln c + \ln \frac{4K_3k_2}{[H^+]H} \left\{ \frac{k_{-2}k_{-3}}{k_3k_4}[H^+] + \frac{k_{-2}}{k_3}(Cu)\,(1-\alpha) \right\}$$

Further introducing

$$H = F\ pO_2;\ F = \frac{30.9\ (H^+)}{0.863 + [H^+]}$$

we have:

$$\ln x = \ln c + \ln \frac{4K_3k_2k_{-2}k_{-3}}{k_3k_4} - \ln F - \ln pO_2 + \ln\left\{1 + \frac{k_4(Cu)}{k_{-3}[H^+]}\right\}(1-\alpha) \quad \text{(XIII)}$$

The second term on the left hand side is a constant, common to all experiments, the third and fourth terms are known functions of the experimental conditions. The fifth term is regarded a constant; that was one of the assumptions necessary to solve the mathematical problem. From the definition of t' it is seen that $t - t'$ is the time the reaction from c_0 to c would have taken, had all the copper been in the cuprous state. Therefore, $1-\alpha = \frac{dt'}{dt}$ or, as long as the order of the reaction is regarded zero, $1-\alpha = \frac{t'}{t}$. The procedure has been to use the value t'/t from the initial part of individual runs to calculate $1-\alpha$, and to use this value for the whole run. This is an approximation, but special calculations have shown that the value of y as a function of $\ln x$ is not

Table 1. Experimental conditions and results for 21 experiments. β is the slope of the (y, t') plots. The last column confirms that these slopes are proportional to the total copper concentration, inversely proportional to the hydrogen ion concentration and independent of the oxygen concentration.

[H+]	$c_0 \cdot 10^3$	(Cu) · 10^4	pO_2	$-\beta$	$-\beta \cdot \frac{[H^+]}{(Cu)}$
0.1	5	2	1.00	0.187	93.5
0.1	5	2	0.660	0.174	87.0
0.1	5	2	0.208	0.175	87.5
0.1	5	2	0.104	0.170	85.0
0.1	5	1	1.00	0.092	92.0
0.1	5	3	1.00	0.265	88.3
0.1	5	5	1.00	0.435	87.0
0.1	5	1	0.208	0.090	90.0
0.2	5	2	1.00	0.084	84.0
0.2	5	2	0.447	0.0821	82.1
0.2	5	2	0.208	0.0853	85.3
0.2	5	2	0.116	0.0862	86.2
0.2	10	1	1.00	0.0453	90.6
0.2	10	1	0.537	0.0450	90.0
0.2	10	1	0.208	0.0430	86.0
0.3	5	2	1.00	0.0597	85.5
0.3	5	2	0.0208	0.0605	90.7
0.3	5	2	0.117	0.0590	88.5
0.4	5	2	1.00	0.0443	88.6
0.4	5	2	0.208	0.0430	86.0
0.4	5	2	0.105	0.0470	94.0

seriously sensitive to the variations of $1-\alpha$ during an experiment, so the credibility of the investigation is not influenced.

The method was to determine a set of values of lnR which gave oxygen independent slopes for the last three runs in Table 1. Afterwards these values were slightly adjusted to give proper copper concentration dependence of the slopes of the four experiments with hydrogen ion concentration 0.1 *M* and oxygen pressure of 1.00 atmosphere. As independent checks (apart from the linearity of the y, t' plots), consistency was found in the determination of the term $k_4(Cu)(1-\alpha)/k_{-3}[H^+]$ from the four experiments mentioned, and universal agreement between theory and experiment as far as dependences on concentrations of hydrogen ion and copper ion of the y, t' slopes are concerned, as demonstrated in Table 1. A further decisive test is reported at the end of the present paper.

The equation for determination of ln$x$ derived was:

$$\ln x = \ln\ 10^3\ c + 0.72 - \ln F - \ln pO_2 + \ln\left\{1 + 330\ \frac{(Cu)}{[H^+]}\ (1-\alpha)\right\} \qquad \text{(XIV)}$$

Table 2 gives the details of the calculation of an experiment, and the linearity of the corresponding y, t' plot is demonstrated on Fig. 2.

Table 2. Details of the first experiment in Table 1. By introduction of the values for concentrations it is found that $H = 3.21$, and by application of the value $(1-a) = 0.78$ further that $\ln x = \ln c \cdot 10^3 - 0.04$ (XIV).

t	$10^3 \cdot c$	$(c_0 - c) \cdot 10^3$	$\frac{c_0 - c}{H\ (Cu)}$	t'	$\ln c \cdot 10^3$	$\ln x$	y	$1-a$
0	4.91	—	—		1.59	1.55	5.42	
3.5	4.46	0.45	0.70	2.80	1.50	1.46	5.12	0.80
6	4.06	0.85	1.32	4.68	1.40	1.36	4.77	0.78
9	3.64	1.77	1.98	7.02	1.29	1.25	4.43	0.78
12	3.13	1.78	2.77	9.23	1.14	1.10	3.98	0.77
15	2.74	2.17	3.38	11.62	1.01	0.97	3.60	
18	2.35	2.56	3.99	14.01	0.85	0.81	3.15	
21	2.01	2.90	4.52	16.48	0.70	0.66	2.74	
24	1.67	3.24	5.05	18.95	0.51	0.47	2.22	
27	1.36	3.55	5.53	21.47	0.30	0.26	1.68	
30	1.14	3.77	5.87	24.13	0.13	0.09	1.25	
33	0.90	4.01	6.25	26.75	−0.10	−0.14	0.77	
36	0.74	4.17	6.50	29.50	−0.30	−0.34	0.26	
39	0.58	4.33	6.74	32.26	−0.54	−0.58	−0.26	
42	0.40	4.44	6.92	35.08	−0.75	−0.79	−0.71	
45	0.35	4.56	7.10	37.90	−1.05	−1.09	−1.38	

From (XIII) and (XIV) we derive the following equations:

$$\frac{4K_s \cdot k_2 \cdot k_{-2} \cdot k_{-3}}{k_3 \cdot k_4} = 10^3 \cdot e^{0.72} = 2.05 \cdot 10^3$$

$$\frac{k_4}{k_{-3}} = 330 \qquad \text{(XV)}$$

Table 1 shows:

$$-\beta \frac{[H^+]}{(Cu)} = 2 \cdot K_s \cdot k_2 = 88$$

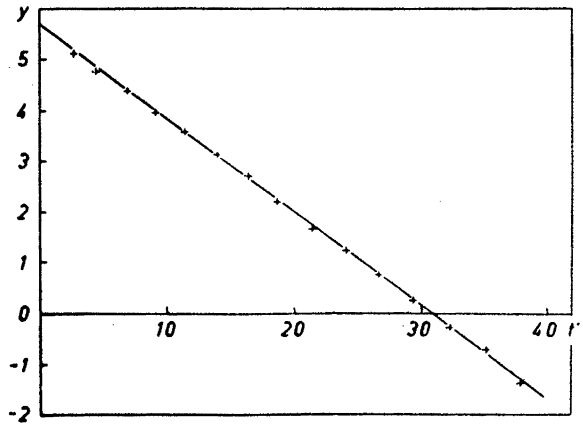

Fig. 2. Demonstration of the linearity of y as a function of t' (see text and Table 2).

The first dissociation constant for ascorbic acid, K_s, is about $6.2 \cdot 10^{-5}$, so we have

$$k_2 \sim 7 \cdot 10^5 \text{ liter mole}^{-1}\text{min}^{-1} \text{ or } k_2 \sim 10^4 \text{ liter mole}^{-1} \text{ sec}^{-1} \quad \text{(XVI)}$$

and $$\frac{k_{-2}}{k_3} \sim 4 \cdot 10^3.$$

(XVI) shows that (+2) is slow compared with (—1) (probable value 10^{11}), so no error was introduced by the simplified calculation of the ascorbic acid ion concentration.

According to a principle pointed out by Christiansen [9] (—2) should be fast. The direct reaction

$$CuCl^-_2 + HA \rightarrow HA^- + Cu^{++} + 2\ Cl^-$$

is probably impossible. To determine the proportion between the genuine constants for (—2) and (+3) we must (the medium being 1 *M* in sodium chloride) divide the value from (XVI) by the mass action constant for the reaction

$$CuCl^-_2 \rightleftharpoons Cu^+ + 2\ Cl^-$$

which is given as $3 \cdot 10^{-6}$,

$$\frac{k_{-2}}{k_3} = \frac{4 \cdot 10^3}{3 \cdot 10^{-6}} \sim 1.3 \cdot 10^9$$

The assumption of low energy of activation for (—2) is justified. It is therefore understandable that the reactions

$$HA \rightarrow H^+ + A^- \qquad (-3)$$

and $$Cu^+ + O_2 \rightarrow CuO_2^+ \qquad (+5)$$

can hardly compete with

$$Cu^+ + HA \rightarrow Cu^{++} + HA^- \qquad (-2)$$

in chloride free medium.

As (+3) is slow (—3) should be fast, which means that HA is a weak acid. The independence of velocity on dehydro ascorbic acid concentration shows that (—4) is slow, so (+4) should also be fast. Therefore k_{-5}/k_4 should not be far from unity. The value 330 is in fair agreement with the difference expected for electrostatic reasons, as an ion with one negative charge should encounter a double charged positive ion more frequently than a single charged ion, other conditions being equal.

The mathematical problem of the present investigation was solved by derivation of the distribution of copper on the two oxidation states. The absorption of cupric ion at 800 mμ is just sufficient to allow a reasonable estimate at concentrations around 10^{-4} when 10 cm cells are used in a Beckman DU spectrophotometer. It must however be emphasised that the determination is rather coarse as the extinctions measured are from about 0.06 downwards.

Table 3. D means the extinction of the reacting solution at 800 mμ in 10 cm cells. The value for a corresponding solution without ascorbic acid was 0.076.

t	H_2A	pO_2	α calc.	D	α obs.
0	4.93	1.00			
5	4.25	1.00			
10	3.45	1.00			
13	3.03	0.80	0.25	0.058	0.24
16	2.71	0.59	0.27	0.055	0.28
19	2.41	0.49	0.30	0.054	0.29
22	2.14	0.35	0.32	0.051	0.33
25	1.93	0.25	0.36	0.047	0.38
28	1.76	0.17	0.41	0.042	0.45
31	1.66	0.12	0.46	0.037	0.52
34	1.58	0.08	0.52	0.032	0.58
37	1.50	0.04	0.61	0.027	0.64
40	1.45	0.02	0.74	0.021	0.72
43	1.42	0.01	0.78	0.017	0.78

An experiment was performed as follows: 25 ml of a solution of ascorbic acid (Co = $5 \cdot 10^{-3}$, (Cu) = $2 \cdot 10^{-4}$, $[H^+] = 0.1$) was placed in the reaction vessel, and oxygen was bubbled through for 10 minutes. Then the solution was quickly transferred to the absorption cell which was filled up into the neck so that there was no gas phase when the glass stopper was inserted. The extinction at 25° C was measured every third minute. From the extinctions the concentrations of cupric copper was determined, and α computed. In a similar kinetic run the bubbling through of oxygen was discontinued after 10 minutes, and the solution was left in the reaction vessel so that dissolved oxygen was allowed to react with the ascorbic acid. The course of the reaction was followed in the usual way (see the experimental section). Stirring of the solution by extraction of samples was carefully avoided.

Introduction of the numerical values for concentrations and constants into (X) gives:

$$\frac{1}{\alpha} = 1 + 3.67 \cdot \frac{pO_2}{c \cdot 10^3}\left\{1 + \sqrt{1 + 0.64 \cdot \frac{c \cdot 10^3}{pO_2}\,[1+0.66(1-\alpha)]}\right\}$$

c was determined by experiment, pO_2 was calculated from stoichiometry. Hence
α could be calculated by iteration. Table 3 demonstrates the accordance between
α calculated in this way and determined by spectrophotometry.

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On the Isomerism of Hydroxyurea

V. Polarographic Behaviour of the Lower-Melting Isomer

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The lower-melting hydroxyurea (m.p. 72 °C) has been found to produce a single, well-developed and reproducible diffusion-controlled cathodic wave at approximately −1.5 V (S.C.E., 25° C) in neutral, buffered and unbuffered solutions, 0.1 to 0.2 *N* in potassium ion. In acidic buffers the hydrogen wave interferes, in alkaline solution, pH> 9, the wave is independently developed, but it decreases rapidly with time due to instability of the substance. The variation of wave height with concentration is linear over the range 0.5 to 5 millimoles/l. The observed influence of the pH upon the half-wave potential together with the shape and the height of the wave seem to indicate that hydroxyurea (m.p. 72°) is reduced according to two distinctly different mechanisms, a pH-dependent one in acid solution and a pH-independent one in neutral and alkaline solution, both being irreversible two-electron processes.

A concurrent polarographic examination of the hydroxylammonium ion confirmed that the lower melting hydroxyurea is not identical with hydroxylammonium cyanate.

Since the higher melting isomer (140° C) proved to be polarographically inactive in the same voltage region, the method developed in this paper is an adequate analytical tool for a study of the formation and interconversion of the isomerides.

The polarographic method, when applicable to an organic compound, not only constitutes an analytical tool of high sensitivity, but very often valuable structural inferences may be drawn from the experimental data. Both potentialities are relevant to the studies dealt with in the present series, the former with a view to a kinetic examination of interconversions of the isomers. This will be the subject of a separate paper. In the following a general polarographic examination of the hydroxyureas is presented and the requisite analytical conditions are reported.

Introductory experiments revealed that in neutral unbuffered electrolyte solution (0.5 *M* lithium chloride) the lower melting isomer (m.p. 72°C) produced a cathodic wave at approximately —1.5 V *vs* S.C.E. ("saturated calomel electrode"), whereas the higher melting isomer (m.p. 140° C) gave neither a

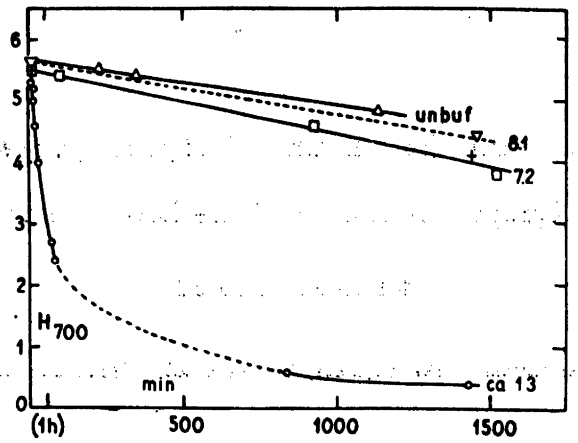

Fig. 1. Lability of hydroxyurea (m. p. 72°) in aqueous solution at room temperature. Wave-height (1 unit = 2.33 microamp.) against minutes from dissolution of the solid. △ 10.0 mM *stock solution (diluted with 4 volumes of buffer at pH 7.2 immediately before recording of the polarogram). 2.0* mM *solutions in buffer pH 7.2 (□), 8.1 (▽), 9.2 (+) and in 0.1* N *potassium hydroxide (○) respectively.*

cathodic nor an anodic wave in the voltage region + 0.2 to —2.0 V (S.C.E.) covered by the experiments. A typical polarographic wave of the lower melting hydroxyurea is given in Fig. 2, pH 7.2, upper curve. The current-voltage curve produced by the higher melting isomer under similar conditions was identical with that of the supporting electrolyte (lower curve) even at high galvanometer sensitivities. This proves the polarographic inactivity of the higher melting isomer and also indicates that it does not contain detectible amounts of the lower melting isomer, when prepared according to the previously reported directions [1].

EXPERIMENTS ON THE STABILITY OF THE LOWER-MELTING HYDROXYUREA IN SOLUTION

Although no quantitative information is available, the lower melting isomer is known to be relatively labile [2, 3, 4] and at the same time the polarographic method of analysis is a rather slow one, the recording of a polarographic wave requiring 2—3 minutes at least. It was therefore imperative to ensure that the error due to the drop in concentration during the time of recording did not seriously exceed the normal error in quantitative polarographic analysis.

Some important results are summarized in Fig. 1, giving the wave height against a time basis. A freshly prepared 0.01 *M* aqueous stock solution was kept at room temperature (20° ± 1°). 1 ml samples were withdrawn at intervals, mixed with 4 ml buffer solution at pH 7.2 (see the last section) and polarographed after appropriate deaeration. The experimental points (△) indicate a drop of approximately 1 % during the first hour from the preparation of the stock solution. This is within the probable error in wave heights (2—4 %).

The first analytical dilution in the above experiment was set aside at room temperature and measured from time to time, Fig. 1, □. It is seen that the stability of this 2.0 m*M* solution in buffer at pH 7.2 is very nearly the same as that of the unbuffered stock solution. Similar orienting experiments were carried out at pH 8.1 (▽) and 9.2 (+) with similar results. The deviations between these curves are probably not significant.

In strongly alkaline medium the situation is different. If a freshly prepared 2.0 m*M* solution in 0.1 *M* potassium hydroxide (pH *ca.* 13) is kept at room temperature, the wave height (Fig. 1, ○) is reduced to approximately half the initial value in the course of the first hour.

In acid solution the lower melting hydroxyurea is perfectly stable. A 10.0 m*M* solution in 0.52 *N* hydrochloric acid was stored in a closed vessel at room temperature. 0.2 ml samples were withdrawn at intervals, diluted with 5 ml buffer to give a final pH of ca. 7 and polarographed under the standard conditions specified in the last section ($s = 150$, $t = 3.2$ sec.). The results are given in Table 1, τ being the time elapsed from the dissolution of hydroxyurea in the hydrochloric acid and until the recording of the polarogram. There is no detectible drop in wave height i_d over a period of 72 hours, on the contrary there appears to be a slight increase. A satisfactory explanation of this phenomenon has not so far been found.

A rough test was made of the lability of neutral solutions at higher temperatures. 5 ml samples of a 10.0 m*M* solution in 0.5 *M* lithium chloride were exposed to heat as indicated in Table 2 and subsequently polarographed at room temperature. *H* is the wave height in arbitrary units.

Table 1.

Stability of hydroxyurea (m.p. 72°) in acidic medium

τ min.	i_d μamp.
5	3.0
13	3.0
25	3.0
37	3.0
79	3.1
96	3.1
116	3.1
153	3.1
205	3.1
280	3.1
365	3.3
4 320 (72 h)	3.2

Table 2.

Thermolability of hydroxyurea (m.p. 72° C) in aqueous solution

	H
not heated	34
heated to the boiling point, quickly cooled	32
boiled for 2 minutes	17
boiled for 5 minutes	5
boiled for 10 minutes	0

The hydroxyurea wave rapidly decreases. After 10 minutes boiling no trace of the original substance is detectible even when applying high galvanometer sensitivity.

To summarize the above results it has been shown that acidic aqueous solutions of the lower melting hydroxyurea are perfectly stable at room

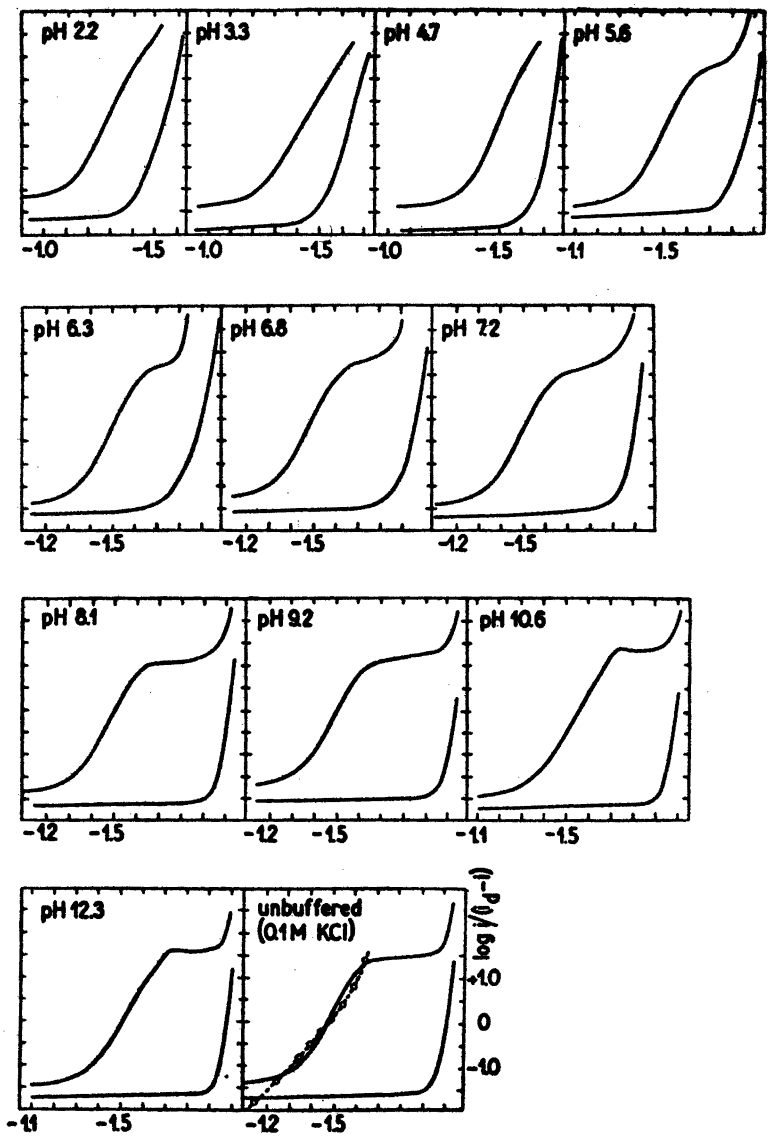

Fig. 2. Polarographic wave of hydroxyurea (m.p. 72°) at various pH values; 2.0 mM *solutions in buffers approximately 0.1—0.2* N *in potassium ion (upper curve). Blank polarograms of the supporting buffers (lower curve). The potential on the abscissa is in volts* vs. *S. C. E., one unit on the ordinate represents 2.33 microamps.*

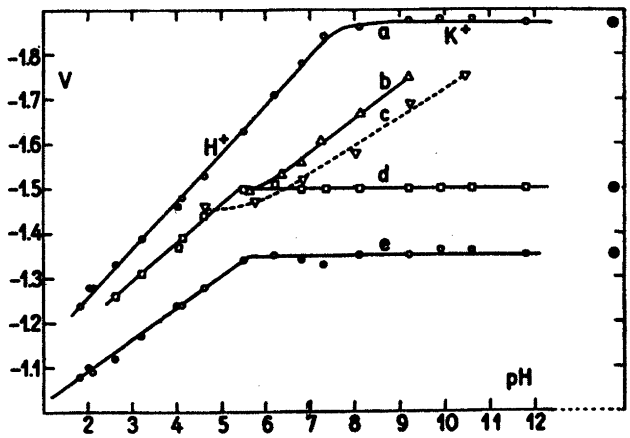

Fig. 3. Variation of half-wave and tangent potentials with the pH. a. tangent potential of the supporting buffer solution. b. 2.0 mM *hydroxylammonium chloride in buffers. Half-wave potential. c. as b., data reported by Petru (Ref.[5] p. 626) 1.0* mM*/ d. 1.0* mM *hydroxyurea (m. p. 72°) in buffers, half-wave potential. e. as d. but tangent potential. The full dots refer to an unbuffered 1.0* mM *solution of hydroxyurea in 0.1* N *potassium chloride.*

temperature. In neutral and slightly alkaline, buffered and unbuffered solutions it is reasonably stable for experimental work carried out within a few hours from the dissolution, but freshly prepared solutions should always be used. Satisfactory stability was also found under conditions extant in polarographic work, *i.e.* in the presence of mercury and maximum suppressor (methylcellulose). Strongly alkaline solutions, pH > *ca.* 11, are very unstable at room temperature and at higher temperatures this also applies to neutral solutions.

The actual process responsible for the deterioration of hydroxyurea (m.p. 72° C) in neutral and alkaline solutions and the kinetics of this reaction from more exact data will be considered in a subsequent paper.

THE INFLUENCE OF THE pH ON THE WAVE OF HYDROXYUREA (m.p. 72° C)

A 10.0 m*M* solution of hydroxyurea in redistilled water was freshly prepared and kept at room temperature. 1 ml samples of this stock solution were mixed with 4 ml of buffer solution and polarographed at 25.0° C after appropriate deaeration. The general technique and the composition of the buffers are given in the last section.

The appearance of the hydroxyurea wave at various pH-values is illustrated in Fig. 2. The lower curve in each graph is that of the supporting buffer solution, polarographed under identical conditions immediately before the hydroxyurea was added. The rise of these blank curves represents the hydrogen discharge in the acidic buffers and the reduction of the supporting potassium ion in the neutral and alkaline buffers.

On the acid side the hydroxyurea wave is poorly developed, it is more or less masked by the hydrogen wave. Measurements of the independent wave can be undertaken with fair accuracy from pH 5.6 onwards, more accurately still from pH 7 and far into the alkaline region. However, in view of the notable instability of hydroxyurea in alkaline solutions, demonstrated in the preceding section, pH 7—8 should be preferred for analytical work.

It should be noted that the wave height is essentially the same over the pH-range investigated and that only one wave appears.

In neutral unbuffered solution with potassium chloride as a supporting electrolyte, last graph in Fig. 2, the wave is of a similar shape and appears to be equally reproducible.

The variation of the half wave potential $E_{0.5}$ with the pH is demonstrated in Fig. 3, curve d (the experimental data are taken from a series different from the one illustrated in Fig. 2). $E_{0.5}$ is shifted to more negative values with rising pH until at pH 5.5 it becomes constant. The implications of this result is discussed later in this paper.

THE INFLUENCE OF CONCENTRATION ON THE WAVE OF HYDROXYUREA

As a consequence of the results reported in the preceding section the buffer having pH 7.2 was chosen for a detailed investigation of the concentration influence. From a 2.0 mM stock solution in the buffer a series of dilutions were prepared as indicated in Table 3 column 2, using the buffer as a diluent. Each

Table 3 (cf. Fig. 4)

Polarogram No.	c millimoles/l	s	H cm	i_d microamp.	E volt
26/7/19	2.0	700	6.9	16.1	−1.45
21	0.01	15	wave undeveloped		
23	0.02	30			
25	0.04	50	2.7	0.5	−1.42 *
26	0.1	50	5.2	0.9	−1.43 *
27	0.2	100	4.9	1.6	−1.43 *
28	0.4	150	7.5	3.8	−1.46
31	0.6	300	5.1	5.1	−1.46
32	0.8	500	4.0	6.7	−1.46
33	1.0	500	4.9	8.2	−1.46
35	1.2	500	5.9	9.8	−1.46
36	1.4	500	6.9	11.5	−1.46
37	1.6	500	7.8	13.0	−1.46
38	1.8	700	6.3	14.7	−1.47
39	2.0	700	6.8	15.9	−1.45
40	2.0	700	6.9	16.1	−1.44
41	3.0	1 000	7.2	24.0	−1.45
42	4.0	1 500	6.4	32.0	−1.45
43	5.0	2 000	6.0	40.0	−1.44
44	6.0	2 000	7.1	47.3	−1.44
45	8.0	3 000	6.1	61.0	−1.45
47	10.0	3 000	7.5	75.0	−1.45

* wave poorly developed.

dilution was deaerated by passing a nitrogen stream through it for 6 minutes, whilst at the same time the following dilution was being prepared and the foregoing one was being recorded. In that way the error due to the lability was reduced to a minimum. Polarogram No. 39 served as a control. It was recorded with the same cell-solution as No. 19 at the end of the series, 2 hours after the preparation of the stock solution. Comparison of the diffusion currents shows that the error due to the lability of hydroxyurea is less than 1.5 % in all the i_d-values.

The higher concentrations were investigated in a separate series, polarograms Nos. 40 to 47. The dilutions were prepared from a fresh 10.0 mM stock solution of hydroxyurea and the operations were carried out exactly as above. The last polarogram in this series was recorded less than one hour from the dissolution of the solid.

In the table s is the current multiplier, H is the wave-height actually measured at the applied galvanometer sensitivity (full sensitivity divided by s), i_d is the diffusion current calculated from the wave-height by means of the expression $i_d = Hs/300$ and $E_{0.5}$ is the half-wave potential in volts *vs* S.C.E., corrected for the iR drop across the polarographic cell, as indicated in the last section.

Apart from the most dilute solutions in which the wave was poorly developed and the experimental error abnormally large, the half-wave potential is virtually independent of concentration. The best value is probably 1.46 ± 0.02 V.

The relation between wave-height (diffusion current) and concentration is graphically represented in Fig. 4. It is perfectly linear up to 5 millimoles/l but shows a slight deviation from linearity above this concentration. The optimal concentration region for analytical work is from 0.5 to 2 millimoles/l, provided the supporting electrolyte is approximately 0.1 M. At higher concentrations the buffering capacity is inadequate, and this cannot be improved by increasing the buffer concentration because the hydrogen wave and potassium wave would then start at a more positive voltage and interfere with the hydroxyurea wave. Concentrations below 0.5 millimoles/l are inconvenient because much greater attention must then be given to the removal of oxygen from the solution.

The slope of the straight line in the optimal concentration region is 8.0. Concentrations may therefore — under standard conditions, see Fig. 4 — be calculated from the experimental diffusion currents i_d by means of the equation $i_d = 8.0\ c$, in which c is given in millimoles/l and i_d in units of microamperes (10^{-6} amp.).

Fig. 5 illustrates the variation of wave-height with concentration in unbuffered solution. 0.5 M lithium chloride was used as a supporting electrolyte. There is strict linearity over the concentration range 0.4 to 10 millimoles/l. The ordinate is the wave-height in cm for a galvanometer sensitivity reduced 1 : 30, but these data were measured some years ago with an older polarographic instrument about 20 times less sensitive, and the results are therefore not directly comparable with other ones reported in this paper. They do, however, support the view that buffering of the cell solution is not strictly necessary in analytical work with hydroxyurea in the neutral region.

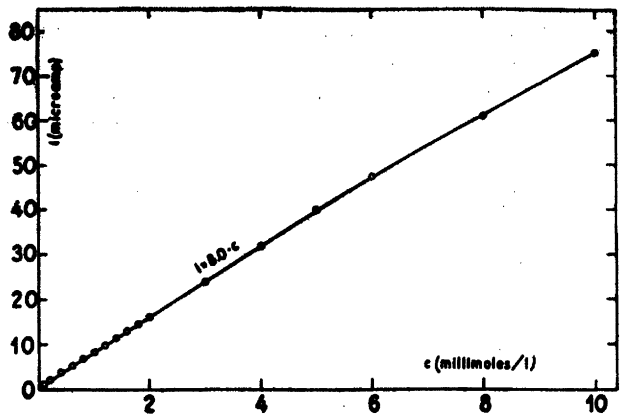

Fig. 4. Hydroxyurea, m. p. 72°. Relation of diffusion current to concentration. Supporting electrolyte 0.1 M *potassium phosphate buffer at pH 7.2. Temp. 25.0° C, drop time* t = *3.33 sec, height of mercury column* h = *45 cm, rate of mercury outflow* m = *2.66 mgsec*$^{-1}$.

THE ELECTRODE PROCESS

The conventional logarithmic analysis of the current-voltage curve of the lower melting hydroxyurea clearly indicates an irreversible electrode process. The general equation of a wave due to a cathodic, *reversible* electrode reaction in a well buffered solution is [6]

$$E = E_{0.5} - \frac{0.0591}{n} \log \frac{i}{i_d - i}$$

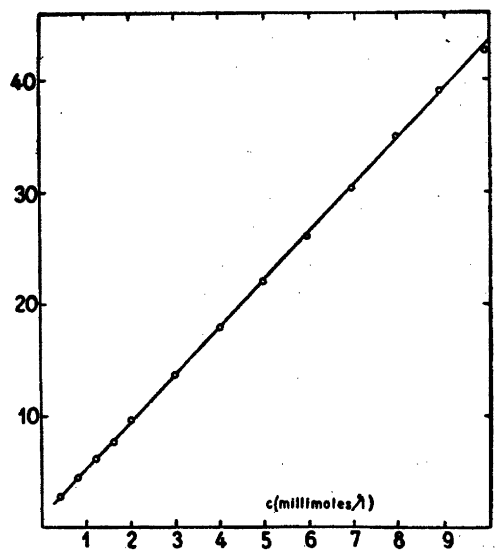

Fig. 5. Hydroxyurea, m. p. 72°. Relation of diffusion current to concentration. Supporting electrolyte 0.5 N *lithium chloride. 20° C.*

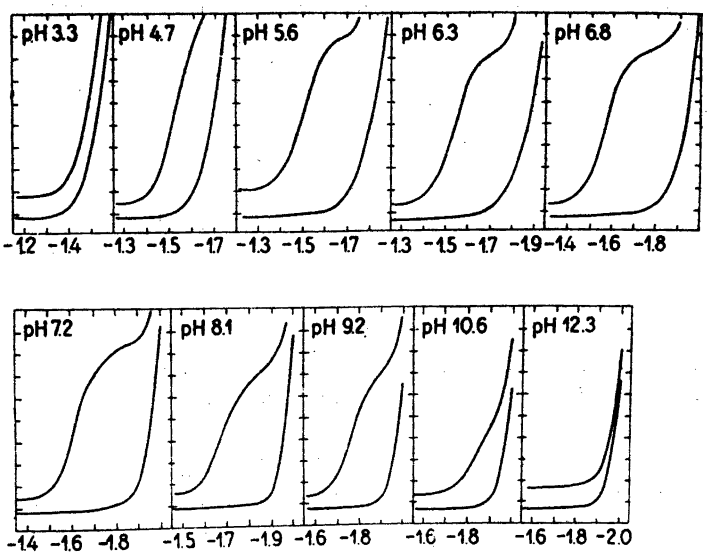

Fig. 6. Polarographic wave of hydroxylammonium chloride at various pH values. 2.0 mM *solutions in buffers approximately 0.1* N *in potassium ion (upper curve). Blank polarograms of the supporting buffers (lower curve). Units and experimental conditions as in Fig. 2.*

i being the diffusion current at any time during the reduction, i_d the limiting diffusion current and n the number of electrons. It follows that a plot of log $\frac{i}{i_d - i}$ against the potential E of the dropping electrode should be linear.

Since n has been estimated to 2 (see below) the theoretical slope would be approximately —30 mV. The actual plot (Fig. 2, last graph) is not linear and the slope in the half-wave point is approximately —190 mV, and thus considerably at variance with the theoretical value. This indicates the irreproducibility of the process.

Since the diffusion coefficient of the lower-melting hydroxyurea is unknown, the number of electrons involved in the cathodic reduction can only be estimated by comparison of the wave height with that produced by some suitable substance, for which the electrode process is established. Hydroxylammonium chloride was chosen for this purpose, firstly because it has a similar molecular size, secondly because it is one of the starting materials in the preparation [1] of hydroxyurea and hence may possibly occur as an impurity, and thirdly because a comparison with the hydroxylammonium wave might provide additional evidence as to whether the lower-melting hydroxyurea is identical with hydroxylammonium cyanate or not.

Although some previous information on the polarographic behaviour of hydroxylammonium chloride is available [5] it was deemed necessary to reinvestigate the substance under conditions strictly identical with those applied to hydroxyurea in Fig. 2.

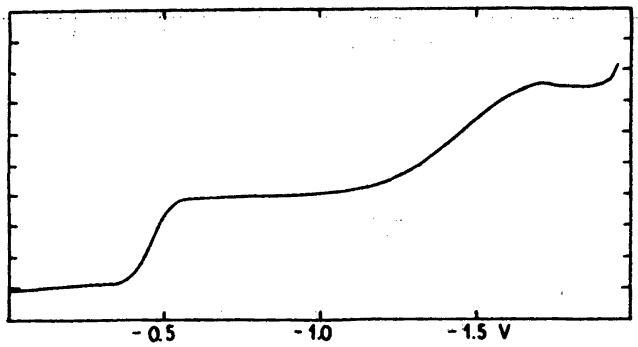

Fig. 7. Polarogram of a solution 1.0 mM *in hydroxyurea, 1.0* mM *in thallous ion and 0.1* M *in potassium chloride. 0.02 % methylcellulose added as a maximum suppressor.*

Freshly prepared 2.0 m*M* solutions of hydroxylammonium chloride in a series of buffers (*cf.* p. 469) were polarographed with the standard technique described in the last section. The results, which on the whole confirm those of Petru [5], are reproduced in Fig. 6. There is a relatively well developed wave in the pH-range from about 6 to 7; in acid solution the wave is masked by the hydrogen wave, in alkaline medium the wave due to the discharge of the supporting potassium ion interferes, in contradistinction to the hydroxyurea wave, which is independently developed far into the alkaline region (*cf.* Fig. 2). In neutral solution, where the wave is well developed, its height is very nearly identical with that of the hydroxyurea wave under equal conditions, *cf.* Fig. 2. Since, according to Petru [5] hydroxylamine is reduced by a two-electron process, it is a reasonable assumption that the cathodic reduction of hydroxyurea also involves two electrons per molecule and this seems to apply over the entire pH-range investigated.

A calculation of n from the Ilkovic equation, using an estimated value of the diffusion coefficient also gave a result close to 2. From polarogram No. 43 (Table 3) the following set of experimental data were drawn; diffusion current $i_d = 40.0$ microamperes, concentration $c = 5.0$ millimoles/l, mercury flow rate $m = 2.66$ mg/sec and drop time $t = 3.33$ sec, temperature 25° C. By inserting these figures in the Ilkovic equation $i_d = 607 \cdot n \cdot D^{1/2} \cdot c \cdot m^{2/3} \cdot t^{1/6}$ we get at 25° C $nD^{1/2} = 0.0056$. The diffusion coefficient D of most organic compounds is of the order of magnitude 1×10^{-5} cm^2 sec^{-1} which gives a value $n = 1.75$.

Finally the wave height of hydroxyurea has been compared with that produced by thallous ion, which is known to react according to a one-electron process. Fig. 7 shows a polarogram of a solution containing equimolar amounts of hydroxyurea and thallous ion. The limiting diffusion currents are 8.4 and 6.3 microamperes, respectively. Since the two species are polarographed under strictly identical conditions we get from the Ilkovic equation

$$\frac{(i_d)_{hy}}{(i_d)_{Tl^+}} = \frac{n_{hy} D_{hy}^{1/2}}{n_{Tl^+} D_{Tl^+}^{1/2}}$$

the index hy standing for hydroxyurea. The diffusion coefficient of thallous ion is $D_{Tl^+} = 2 \times 10^{-5}$ cm^2 sec^{-1}. Introducing this, as well as $n = 1$, the above experimental diffusion currents and the assumed diffusion coefficient $D_{hy} = 1 \times 10^{-5}$ cm^2 sec^{-1} we get $n_{hy} = 1.7$. There seems to be every reason to believe, therefore, that the reduction of hydroxyurea, m.p. 72°, involves two electrons per molecule.

In the dependency of the half wave potential upon the pH hydroxylamine and hydroxyurea show a remarkable difference (Fig. 3, b and d). Whereas $E_{0.5}$ of the former is strongly dependent upon the pH in the region pH 5—10 that of the latter is constant in the same region. The $E_{0.5}$ values for hydroxylamine are somewhat larger than those reported by Petru [5] (Fig. 3 c) but it should be remembered that the absolute values can only be roughly estimated because the wave is more or less masked by the hydrogen wave in all the buffer solutions.

For hydroxyurea the relation between the half-wave potential and the pH has the form of two straight lines intersecting at pH 5.5. The linear dependency in acid solution is substantiated by supplementary measurements (e) of the tangent potential (45°) (Ref.[6], p. 623) in strongly acidic medium, where the half-wave potential cannot be determined with any great accuracy. The general relation between $E_{0.5}$ and pH for a *reversible* reduction $R + nH^+ + ne^- \rightleftharpoons RH_n$ is given by [6]

$$E_{0.5} = E^\circ_{0.5} - \frac{0.0591}{n} \log \frac{k_R}{k_{RH_n}} - 0.0591 \text{ pH} \qquad (1)$$

in which $E^\circ_{0.5}$ is the standard potential of the reaction and k a constant characteristic of the species. The second term on the right is usually negligibly small. It follows that the theoretical shift of the half-wave potential due to one pH-unit is —59 millivolts for the above *reversible* reaction. The acidic branch of the curve Fig. 3 d is strictly linear as required, but its slope is —87 millivolts.

The slope —87 millivolts is so close to the average slope found for the discharge of the hydrogen ion (Ref.[12], p. 123; *cf.* Fig. 3 a) that the possibility should be considered whether the hydroxyurea wave in acid medium be due to hydrogen discharge from the hydroxyuronium ion functioning as a weak acid, the discharge for some reason taking place slightly before the hydrogen wave of the buffer starts. This explanation is, however, unlikely, because the hydrogen wave is much higher than that due to normal organic compounds. Consequently a considerable change in wave height should have been observed for hydroxyurea when shifting from acid to alkaline medium. As reported above (*cf.* Fig. 2) no such change was observed, the wave height is constant over the entire pH-range.

The deviation of the slope from the theoretical value may then be explained either as a result of the irreproducibility of the process or by assuming an anomalous number of hydrogen ions to take part in the electrode process [12]. Equation (1) is valid only for a process involving the same number (usually $n = 2$) of electrons and hydrogen ions. If the numbers were n and m respectively, the constant in the last term in eq. 1 would be $\frac{m}{n}$ (—59) mV. It is seen that

an electrode process involving two electrons and three hydrogen ions per molecule

$$R + 2e^- + 3H^+ \rightarrow RH_3^+ \qquad (2)$$

would give a shift of $3/2 \cdot (-59) = -88$ mV in close agreement with the experimental value.

In neutral and alkaline solution the half-wave potential of hydroxyurea is completely independent of the pH. The distinct break at pH 5.5 suggests that hydroxyurea is reduced at the dropping mercury-electrode by two distinctly different mechanisms. A pH-dependent one, in which hydrogen ions take part in the potential-determining step, operates in acid solution. As the hydrogen ion activity decreases, the reduction by this mechanism finally becomes so difficult (*cf.* the rising $E_{0.5}$) that a different, pH-*in*dependent mechanism is favoured. That hydrogen ions do not take part in the second mechanism seems to be substantiated by the fact that hydroxyurea produces a well-defined wave of exactly the same height in neutral *unbuffered* solutions (*cf.* Fig. 2, last graph) in distinct contrast to hydroxylamine, which gives ill-defined double waves under such conditions, as expected with the mechanism suggested by Petru [5] involving hydrogen ions. It is quite certain, therefore, that in neutral and alkaline medium hydroxyurea and hydroxylamine are reduced by entirely different mechanisms and the question as to whether the lower-melting hydroxyurea is simply hydroxylammonium cyanate has at the same time received a clear negative answer. Whether the pH-dependent mechanisms are of a similar type remains to be settled.

The regions of pH-dependency fall in the vicinity of the dissociation constants, $pK_a = 8.0$ for hydroxylammonium ion [10] and ca. 2.3 for the hydroxyuronium ion [11]. It seems probable that the species, which is reduced at the dropping electrode in the case of hydroxyurea is the uncharged basic molecule (B) in neutral and alkaline medium and the conjugate acid, hydroxyuronium ion (BH^+) in acid solution. The fact that only a single wave of constant height is obtained over the whole pH-range indicates that the protolytic reaction $BH^+ \rightleftharpoons B + H^+$ proceeds with infinite velocity (*cf.* Ref.[7]) so that the hydroxyuronium ions consumed by the electrode process are immediately replaced by newly formed ones. Under these circumstances (*cf.* Ref.[8]) no separate reduction of the basic form takes place until at about pH 5.5 the pH-dependent mechanism has become so difficult that the pH-*in*dependent reduction of the uncharged molecule, taking place at a slightly more negative potential, is favoured. As a result of these combined mechanisms the limiting current (wave height) would be independent of pH and proportional to the total analytical concentration of hydroxyurea, as indeed we find.

The possibilities that the limiting current is either kinetically controlled or of a catalytic nature have been ruled out since the relation $i_d = k \cdot h^{1/2}$ (Ref.[6], p. 85) characteristic of a purely diffusion-controlled current has been found to hold for hydroxyurea. Table 4 contains the experimental material. h_{corr} is the height of the mercury column, corrected for the back-pressure $-\frac{3.1}{(mt)^{1/3}}$ due to interfacial tension (Ref.[6], p. 79) which for the employed capillary ($mt = 7.68$ mg) amounted to -1.6 cm Hg.

Table 4. Variation of i_d *with the height* $h_{corr.}$ *of the mercury column. 2.0* mM *hydroxyurea in 0.1* N *potassium chloride, 0.02* % *methylcellulose.*

$h_{corr.}$ cm	t sec	i_d μamp	$i_d / h_{corr}^{1/2}$
28.4	3.7	14.5	2.72
38.4	2.8	16.6	2.68
43.4	2.5	17.8	2.70
48.4	2.2	18.7	2.69
58.4	1.9	20.3	2.67
68.4	1.6	22.0	2.66

The values in the last column are virtually constant. The apparent decrease is due partly to the approximative nature of the equation $i_d = k \cdot h^{1/2}$ (Ref.[5], p. 85) and partly to the lability of the substance in aqueous solution. The last polarogram in Table 4 was recorded 1 ½ hour after the first one. According to previous results a drop of 1—2 % in the "constant" is to be expected in this period, due to the lability of hydroxyurea. Much larger deviations in $i_d/h_{corr}^{1/2}$ would have been found if the limiting current were kinetically controlled. Frequently the current is quite independent of the height of the mercury column (Ref.[5], p. 274). Adsorption waves usually show a linear relationship between the limiting current and h.

No attempt will be made at the present time to formulate definite electrode processes. When dealing with irreversible reductions of organic molecules this cannot normally be achieved on the basis of polarographic data alone.

It seems likely, however, that urea must be taken into account as a possible reduction product. This would be compatible with a two-electron process and, since urea has been found to be inactive towards the dropping mercury electrode, also with the apparent irreversibility of the electrode process. It should be added that the reaction scheme (2), p. 466, must be discarded, if the reduction product is shown to to be urea, because this substance is a *weaker* base than hydroxyurea, whereas the hypothetical process

$$O = C\begin{matrix}\diagup NHOH \\ \diagdown NH_2\end{matrix} + 2e^- + 3H^+ \rightarrow O = C\begin{matrix}\diagup NH_3^+ \\ \diagdown NH_2\end{matrix} + H_2O$$

corresponding to (2), requires that the reduction product be a stronger base than hydroxyurea. Isolation of urea from a large scale controlled-potential electrolysis is being attempted.

The higher-melting hydroxyurea (140°) produces neither a cathodic nor an anodic wave in the usual voltage region + 0.2 to —2.0 V *vs* S.C.E. It may be assumed that a much more negative potential is required for the reduction of the higher melting isomer; this would be parallelled by the greater robustness of that isomer. Alternatively the reducible group in the lower melting isomer may be absent in the higher melting one.

Methoxyurea, which only exists in one form, is not reduced at the dropping mercury electrode. This is compatible with the view that the —NHOH group is engaged in the polarographic reduction of the lower melting isomer.

MATERIALS, APPARATUS, AND GENERAL TECHNIQUE

The preparation and characterization of the isomeric hydroxyureas have been reported in a previous paper [1].

The lower melting hydroxyurea was stored at 0° C in a dry atmosphere, and all measurements reported in this paper were carried out within 2 weeks from the preparation. Comparison of the wave-height produced under standard conditions at various instants indicated a satisfactory stability under these circumstances.

Hydroxylammonium chloride as well as all chemicals used for the preparation of the supporting solutions were analytical grade products. The solvent was water, carefully redistilled in a Pyrex apparatus. The composition of the various stock solutions used for preparing the buffers are indicated in Table 5.

Table 5. Composition of buffer stock-solutions.

A	1 *M* phosphoric acid 1 *M* potassium chloride
B	1 *M* citric acid 1 *M* potassium chloride
C	1 *M* acetic acid 1 *M* potassium chloride
D	1 *M* potassium hydrogen carbonate
E	1 *M* boric acid 1 *M* potassium chloride
F	1 *M* potassium di-hydrogen phosphate
G	1 *M* potassium mono-hydrogen phosphate
H	1 *M* potassium di-hydrogen borate
I	1 *M* potassium hydroxide

The set of stock solutions used for each individual buffer is indicated in Table 6. Appropriate volumes were mixed and diluted to the tenfold volume with water containing 0.05 % methylcellulose as a maximum suppressor. In most experiments 4 to 4.5 ml of the buffer were mixed with 1 to 0.5 ml of a stock solution of the depolarizer, so that the final volume of cell solution was 5 ml, and the final concentration of potassium ion was approximately 0.1 to 0.2 *M*. By employing a relatively large number of buffer types (Table 6, col. 4) and using each system only in the immediate neighbourhood of the relevant p*K*-value a sufficient buffer capacity was secured in all solutions. This is a point of great importance often neglected in polarographic work [9]. The final pH of each cell-solution was measured after the polarographic recording by means of a "Radiometer" pH-meter, type PHM 221, against a standard phosphate buffer of pH 6.78.

The polarographic cell was a cylindrical glass tube, 17 by 50 mm, fitting into a brass water-jacket through which water at 25.0 ± 0.1 was circulated from a Höppler ultra-thermostat. The anode was a saturated potassium chloride-calomel electrode with a saturated potassium chloride liquid junction. Unless otherwise stated the capillary characteristics of the dropping mercury electrode were as follows, height of mercury column h = 45 cm, rate of mercury outflow into distilled water m = 2.66 mg sec^{-1}, drop time 3.0 to 3.3 sec.

Table 6.

Final pH	Stock solutions		Buffer type
	acid	base	
1.0	A	F	phosphate
1.6	A	F	
2.2	A	F	
2.4	A	F	
2.9	B	I	citrate
3.6	B	I	
4.0	C	I	acetate
4.7	C	I	
5.6	F	G	phosphate
6.3	F	G	
6.8	F	G	
7.2	F	G	
8.1	F	G	
9.2	E	H	borate
9.7	D	I	carbonate
10.5	D	I	
11.9	G	I	phosphate

Oxygen was removed from the cell-solution prior to any polarographic recording by passing a stream of oxygen-free nitrogen through the cell. 6 to 10 minutes were required to reduce the oxygen wave to a negligible height, depending upon the applied galvanometer sensitivity. The absence of the oxygen wave and of waves due to other impurities was checked by running a blank polarogram of the supporting electrolyte solution before the addition of the depolarizer.

The polarographic instrument was a "Radiometer" PO 3e. The voltage scale was checked against thallous ion, the half-wave potential of which was taken as -0.46 V *vs* S.C.E. Half-wave potentials were corrected for the iR-drop across the cell, when necessary. The resistance of the polarographic cell under average experimental conditions was determined by measurement of a series of widely different concentrations of thallous ion. The plot of the apparent half-wave potentials against the diffusion current in the half-wave point was linear with the slope $\triangle E_{0.5} / \triangle i_{0.5} = 2.0 \times 10^3$, which is equal to R. The correction thus amounts to $+0.002\ i_{0.5}$ volts, $i_{0.5}$ being in units of microamperes. It is seen that the correction is significant only for diffusion currents $i_d > 20$ microamperes ($i_{0.5} > 10$), since the probable error in polarographic half-wave potentials with the employed technique is estimated to $\pm$ 0.02 V.

The full galvanometer sensitivity was maintained at a constant value of 7.8 cm deflection for a current of 2.6×10^{-8} amperes by frequent adjustment. In most measurements this sensitivity was reduced to 1/300 to 1/500 (current multiplyer s = 300 to 500). The diffusion currents i_d were calculated from $i_d = H \cdot s/300$, H being the wave height in cm and i_d the current in microamperes (10^{-6} amperes). The wave-height H was measured as the vertical distance between the tangent intersection points and has thus been corrected for the residual current.

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On the Thermal Decomposition of Molybdenum Trioxide *in vacuo*

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When heated in evacuated, sealed silica tubes at temperatures between 550° and 800° C molybdenum trioxide has been found to undergo partial decomposition forming various lower molybdenum oxides. Among the latter are two previously unknown compounds, ζ- and η-molybdenum oxide, which have been characterized by their X-ray powder patterns.

In connection with studies on various molybdenum oxides carried out at this Institute it was observed that molybdenum trioxide decomposes when heated in evacuated, sealed silica tubes at red heat. After the heat treatment the trioxide crystals were slightly contaminated by dark blue or bluish-violet particles. Previous authors have also reported that molybdenum trioxide is unstable at reduced pressures of oxygen. Thus Nydahl [1] observed that molybdenum trioxide, when sublimed in a stream of air at about 800° C, gives partly reduced bluish products, while preparations obtained at the same temperature by sublimation in oxygen are colourless or faintly yellow. Decomposition of molybdenum trioxide in the electron microscope has been reported by König [2,3], who observed the formation of dioxide during this process, and by Glemser and Lutz [4], who found the decomposition products to contain the oxides Mo_4O_{11}, Mo_8O_{23}, Mo_9O_{26}, and probably MoO_2. Studies on the thermal decomposition of tungsten trioxide have previously been carried out at this Institute [5].

In order to make sure that no reducing material was present, our experiments were carried out using molybdenum trioxide which had been sublimed in a stream of oxygen at about 800° C*. The heat treatment was performed in silica tubes which had been preheated in a stream of oxygen for several hours at 900° C. The reaction tubes containing the molybdenum trioxide samples were evacuated by means of a rotary oil pump, a cold trap with liquid air being attached to the pump, and sealed in a gas oxygen flame.

* A finely ground specimen of this preparation was found to have grown appreciably darker than a freshly powdered sample after preservation for a few months in a covered weighing bottle kept in the laboratory.

The heat treatment was carried out in an electric furnace. In order to reduce the temperature gradient within the reaction tubes they were kept in holes bored in a heavy cylindrical block of fire-resisting steel. This block was covered with a steel lid and vertically inserted in the furnace. A platinum-platinum rhodium thermocouple was introduced in one of the holes of the cylinder through a narrow opening in the lid. The variation of the temperature during an experiment did not exceed $\pm$ 5° C. The samples were quenched in water from the reaction temperature.

The preparations were investigated under the microscope. Concentrated samples of the reduced material were obtained by picking out the dark particles. The phases forming relatively large crystals, *viz.* Mo_8O_{23} and Mo_9O_{26}, were readily isolated in this way, while the enrichment of those occurring in very small crystals offered some difficulties. Attempts to perform the separation of the phases chemically, *e.g.* by means of ammonia, were less successful. The various phases were identified by means of X-ray powder photographs taken in a Guinier focusing camera using Cu-$K\alpha$ radiation. Sodium chloride was always added to the powder specimens as an internal standard ($a_{NaCl} = 5.6398$ Å). A survey of the results of the phase analyses of several preparations is given in Table 1.

Table 1. Phase analysis of preparations obtained by heating molybdenum trioxide in evacuated, sealed silica tubes.

Heating temperature	Heating time	Phases identified by microscopical examination	Phases identified by powder photographs
549° C	87 hours	MoO_3 crystals + small amount of irregular bluish-black aggregates, consisting of extremely thin, fragile plates	MoO_3 + η-oxide
608° C	46 hours	MoO_3 crystals + aggregates of dark crystals	MoO_3 + η-oxide
650° C	44 hours	MoO_3 crystals + prismatic, blue-violet crystals or crystal aggregates of high brilliance	MoO_3 + Mo_8O_{23}
700° C	46 hours	—"— —"— —"—	MoO_3 + Mo_8O_{23}
722° C	42 hours	—"— —"— —"—	MoO_3 + Mo_9O_{26}
803° C	42 hours	Highly discoloured, greyish-black MoO_3. (An extremely small amount of a bluish-black phase isolated by treatment with ammonia)	MoO_3

In all cases the quantity of reduced material in the samples was very small in comparison with the amount of undecomposed trioxide, but generally seemed to increase with increasing heating temperature as long as the melting point of the preparations was not exceeded. The main portion of the reduced substance generally appeared in the top layer of the trioxide samples.

After heating at high temperatures (below the melting temperature) the molybdenum trioxide appeared in discoloured, greyish-blue or green crystals which, however, gave powder patterns very similar to those obtained from trioxide crystals of normal appearance. Thus only some minor variations of the intensities of the lines could be observed which may be attributed to

Table 2. Powder pattern of ζ-molybdenum oxide. ($MoO_{2.88}$ heated at about 600° C for 2 days.)

I	*d* Å	*I*	*d* Å
w	7.25	vw	2.562
w	4.02	vw	2.520
vw	3.91	vw	2.319
s	3.58	vw	2.307
vw	3.51	w	2.299
s	3.36	vw	2.288
s-	3.27	vw (diffuse)	2.169
vw	3.11	vw (diffuse)	2.102
vw	3.05	m	1.989
vw	2.771	w (diffuse)	1.960
vw	2.710	m	1.869
w	2.603		

orientation effects. At present it is not possible to conclude whether the reason for this change of colour is the existence of an extended homogeneity range of the trioxide structure or the formation of another phase (*cf.* the discussion of the thermal decomposition of tungsten trioxide [5]).

Samples heated above the melting temperature formed a greyish-black mass from which it was not possible to separate the constituents by picking out crystals. By treating with dilute ammonia the molybdenum trioxide was dissolved, leaving an extremely small residue of a bluish-black substance. However, the quantity of this material was too small to allow a powder photograph to be taken.

In spite of the care given to the experimental conditions it was very difficult to obtain reproducible results in some cases. Thus some of the preparations obtained definitely did not correspond to a state of equilibrium. A sample heated at 680° C for 72 hours was found to contain MoO_3, Mo_8O_{23} and another oxide phase (ζ-molybdenum oxide) which has also been prepared by heating a mixture of molybdenum and molybdenum trioxide of the gross composition $MoO_{2.88}$ at about 600° C *in vacuo*. In a molybdenum trioxide sample heated at 753° C for 45 hours Mo_9O_{26} as well as Mo_4O_{11} could be identified in the powder

Table 3. Powder pattern of η-molybdenum oxide, obtained by heating molybdenum trioxide in vacuo at 608° C.

I	*d* Å
vw	6.04
s	3.92
m	3.60
w	3.39
vw	3.34
vw	2.811
w	2.717
w	2.669

patterns in addition to the trioxide. Similar difficulties in reaching a state of equilibrium have also been observed previously in connection with attempts to prepare the oxides Mo_8O_{23} and Mo_9O_{26} in a pure state by heating molybdenum metal and trioxide *in vacuo*[6]. Due to the difficulties in performing the phase analyses it cannot at present be definitely concluded whether or not the samples listed in Table 1 correspond to states of equilibrium.

However, the experiments show that molybdenum trioxide undergoes a partial decomposition under the conditions applied. As the presence of reducing material was carefully avoided it seems most likely that the trioxide is unstable in the temperature interval 550°—800° C at a reduced pressure of oxygen. Two previously unknown molybdenum oxides have been observed during the experiments and preliminarily designated with the symbols ζ-molybdenum oxide and η-molybdenum oxide. Powder patterns which are likely to represent fairly pure samples of these two oxides are listed in Table 2 and 3. These serve to complement the powder data on molybdenum oxides previously reported from this Institute[7].

The molybdenum oxides known at present are thus MoO_3[8–10], Mo_9O_{26}[11], Mo_8O_{23}[11], Mo_4O_{11}[12], MoO_2[13], and Mo_3O[14], the crystal structures of which have been determined, and the ε-[5], ζ-, and η-oxides of unknown compositions and structures. Further studies on the molybdenum-oxygen system are in progress.

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Reaction of Furfuryl Alcohol, Furfuryl Methyl Ether and 2,5-Dimethoxy-2,5-dihydrosilvan with Methanolic Hydrogen Chloride

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The products formed by interaction of methanolic hydrogen chloride and furfuryl alcohol, furfuryl methyl ether and 2,5-dimethoxy-2,5-dihydrosilvan, respectively, have been analyzed by fractional distillation. Four new compounds, *viz.* 2,3 (or 4), 5-trimethoxytetrahydrosilvan, methyl levulinate dimethyl ketal, α(or β)-methoxylevulinaldehyde dimethyl acetal and 1,1,2 (or 3), 4,4-pentamethoxypentane were identified and at least three unknown compounds were shown to be present. Earlier experiments in this field, in particular by Pummerer *et al.*, *Ber.* **56** (1923) 999; **68** (1935) 480, are discussed.

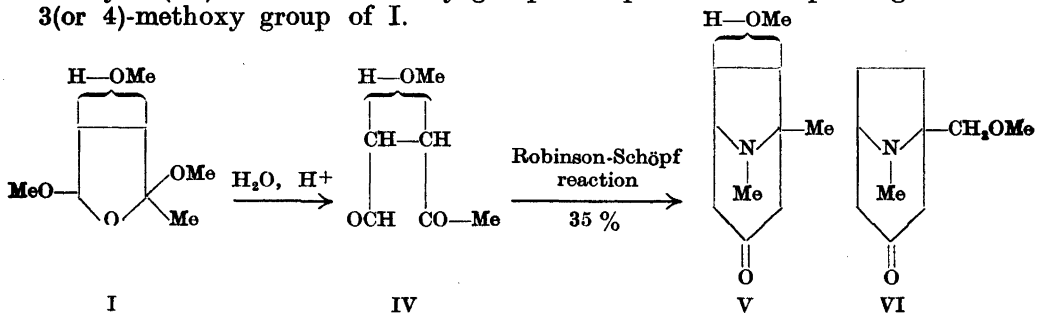
By heating furfuryl alcohol, furfuryl methyl ether and 2,5-dimethoxy-2,5-dihydrosilvan, respectively, under reflux with methanolic hydrogen chloride, distillable mixtures of liquid compounds were obtained and separated by fractional distillation under 21 mm through a 25 cm column (distillation time 8—10 days). The results of the distillations are shown in Fig. 1. The fractions were mainly identified by measuring the refractive index and the infrared absorption, and by analyses. Three new compounds, *viz.* 2,3 (or 4),5-trimethoxytetrahydrosilvan (I), methyl levulinate dimethyl ketal (II) and α(or β)-methoxylevulinaldehyde dimethyl acetal (III) were identified, and at least 3 unknown compounds were shown to be present. The yields of the various compounds are given in Table 1.

I is stable towards alkali and shows no absorption band around 5.8 mμ. Hydrolysis with 0.025 *N* hydrochloric acid gives a reaction product, which condenses with methylamine and acetonedicarboxylic acid (Robinson-Schöpf reaction) to a tropinone $C_9H_{14}ON(OMe)$, which is different from the known 1-methoxymethyltropinone (VI) and therefore is 1-methyl-6(or 7)-methoxytropinone (V). From this, and from the fact that I not only is formed from furfuryl alcohol and furfuryl methyl ether, but also from dimethoxydihydrosilvan, it is inferred that the compound is either a 2,3,5- or a 2,4,5-trimethoxy-

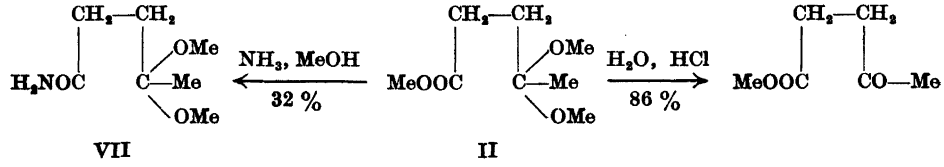
Table 1. Yields of compounds present in the reaction mixtures prepared from furfuryl alcohol, furfuryl methyl ether and dimethoxydihydrosilvan.

Compound	Yield, % based upon			Yield, % of distillable product from		
	Furfuryl alcohol	Furfuryl methyl ether	Dimeth-oxy-dihydro-silvan	Furfuryl alcohol	Furfuryl methyl ether	Dimeth-oxy-dihydro-silvan
I. [2,3 (or 4), 5-Tri-methoxytetrahydro-silvan]	3.0	1.8	2.8	7	5	4
Methyl levulinate	27.7	11.2	15.2	65	32	22
II. (Methyl levulinate dimethyl ketal)	1.2	15.3	33.8	3	44	49
III. [α (or β)-Methoxy-levulinaldehyde dimethyl acetal]	10.1	0.0	0.0	24	0	0
Unknown compounds	0.3	6.2	17.3	1	18	25
Total yield of distillable compounds	42.3	34.5	69.1	100	100	100

tetrahydrosilvan and that the product of hydrolysis is α- or β-methoxylevulinaldehyde (IV) with the methoxy group in a position corresponding to the 3(or 4)-methoxy group of I.



II is transformed into methyl levulinate by heating under reflux with methanolic hydrogen chloride containing 1.07 mole of water. Reaction with ammonia gives a crystalline compound with the formula $C_5H_9ON(OMe)_2$. From this it is inferred that II is methyl levulinate dimethyl ketal and that the nitrogen containing compound is the corresponding amide (VII).



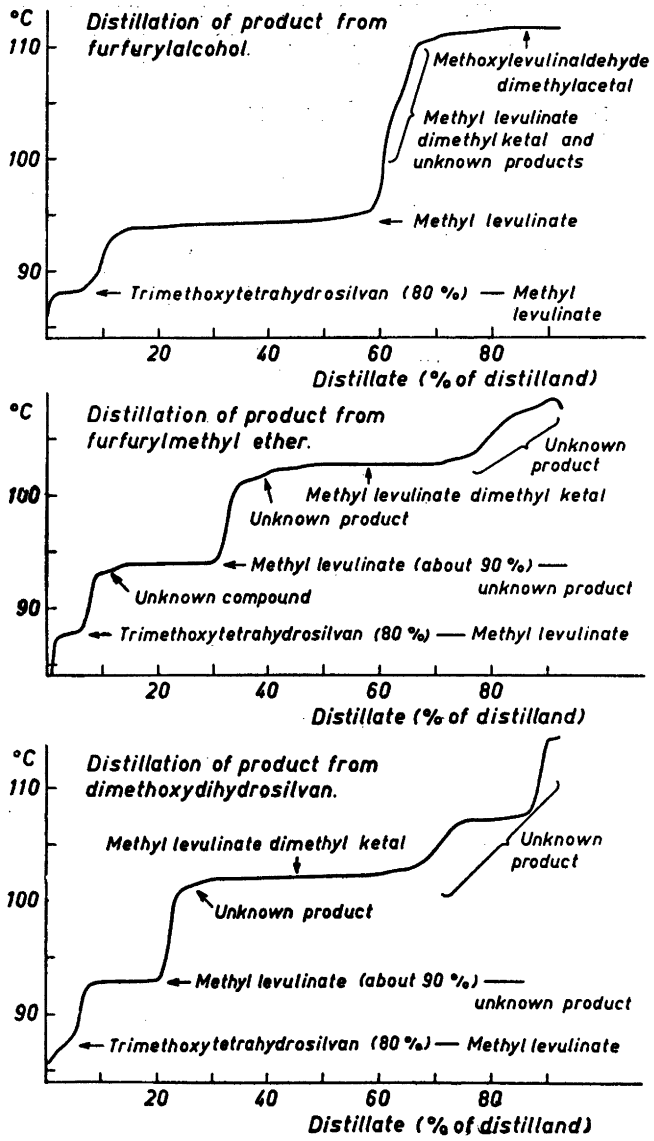
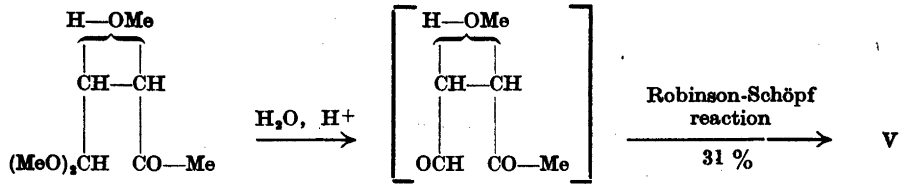


Fig. 1. Curves for the distillations of the reaction mixtures prepared from furfuryl alcohol, furfuryl methyl ether and dimethoxydihydrosilvan.

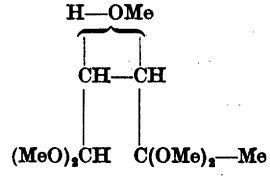
III is unstable towards alkali. Hydrolysis with hydrochloric acid and condensation of the reaction product with methylamine and acetonedicarboxylic acid give a tropinone, which is identical with V. From this it is inferred that the product of hydrolysis is either α- or β-methoxylevulinaldehyde (IV)

and that III is α- or β-methoxylevulinaldehyde dimethyl acetal with the methoxy group in a position corresponding to the position of the 3(or 4)-methoxy group of I.



III IV

The percentage of unknown compounds in the distillate from furfuryl alcohol is small compared to the percentage in the distillates from furfuryl methyl ether and dimethoxydihydrosilvan, respectively. As about 4 % (by weight) of methanol is slowly split off during the distillation of the reaction products from furfuryl methyl ether and dimethoxydihydrosilvan we assumed that a number of the unknown compounds are formed from 1,1,2(or 3),4,4-pentamethoxypentane (VIII) by loss of one or two moles of methanol.



VIII

In order to test this hypothesis, the reaction mixture from furfuryl methyl ether was heated under reflux with a solution of potassium hydroxide in methanol. This should remove all esters and all compounds containing free carbonyl groups. The resulting product was then distilled rapidly under 12 mm through a 12 cm column (distillation time 12 hours). The result of the distillation is shown in Fig. 2 and the yields of the products, based upon furfuryl methyl ether, are given in Table 2.

VIII is stable towards alkali and shows no absorption band around 5.8 mμ. Acid hydrolysis followed by a Robinson-Schöpf reaction gives the tropinone V.

Table 2. Yields of alkali-resistant compounds from furfuryl methyl ether.

Compound	Yield, % based upon furfuryl methyl ether
2,3 (or 4)-Trimethoxytetrahydrosilvan (I)	1.1
Unknown compounds	about 2.5
1,1,2 (or 3), 4,4-Pentamethoxypentane (VIII)	0.7

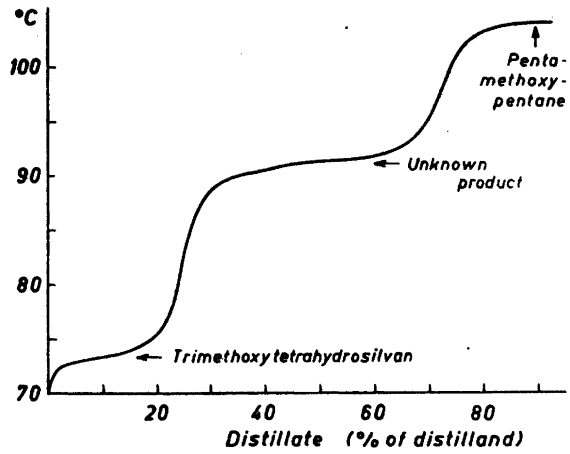


Fig. 2. Curve for the distillation of alkali-treated reaction product from furfuryl methyl ether.

From this it is inferred that the product of hydrolysis is α- or β-methoxylevulinaldehyde (IV) and that VIII is 1,1,2 (or 3),4,4-pentamethoxypentane with the 2(or 3)-methoxy group in a position corresponding to the position of the 3(or 4)-methoxy group of I.

Although the pentamethoxypentane VIII has thus been isolated, the yield is much lower than the yields of unknown compounds from the distillations of the reaction mixtures from furfuryl methyl ether and dimethoxydihydrosilvan. However, we still think that VIII is the progenitor of the major portion of the unknown compounds and that the yield of VIII in the isolation experiment is low, because most of the compound has been hydrolyzed or transformed in other ways during the alkaline treatment and the isolation.

The formation of acetals of α(or β)-methoxylevulinaldehyde (IV) in the reaction of dimethoxydihydrosilvan with methanolic hydrogen chloride is just another example of the known addition of an alcohol to the 3,4-double bond of 2,5-dimethoxy-2,5-dihydrofurans by the action of the alcohol and a catalytic amount of a strong acid [1]. The formation of derivatives of IV from furfuryl alcohol and furfuryl methyl ether may, on the other hand, be taken as proof of the initial formation of dimethoxydihydrosilvan or its equivalent during the fission of furfuryl alcohol and furfuryl methyl ether, respectively. This is consistent with experiments with similar compounds as well as with theoretical considerations [2, 3, 4].

The distillable reaction product from furfuryl methyl ether has by and large the same composition as the product from dimethoxydihydrosilvan. The composition of the product from furfuryl alcohol is somewhat different, but this becomes understandable if one takes into account that in any reaction between furfuryl alcohol and methanol there is an excess of one mole of water over similar reactions with furfuryl methyl ether and dimethoxydihydrosilvan, respectively.

In spite of the similarity in composition, the yield of distillable product from dimethoxydihydrosilvan is almost twice as large as from furfuryl alcohol and furfuryl methyl ether, respectively. This can be understood by assuming that the fission reactions of furfuryl alcohol and furfuryl methyl ether to distillable products compete with other reactions leading to non-distillable products (polymerisation reactions), while the reactions of all the compounds roughly speaking follow the same path after the furans have been rearranged to dimethoxydihydrosilvan or its equivalent.

Pummerer *et al.*[5,6] (*cf.* also [7]) have previously investigated the action of methanolic hydrogen chloride on furfuryl alcohol and furfuryl methyl ether and the reaction conditions given by these authors have been followed here. From the reaction product from furfuryl alcohol they isolated 2 % of impure methyl levulinate and 19 % of a product, which, judging from their experimental data, was a mixture of two or more compounds, but which was claimed to be 5-methoxylevulinaldehyde dimethyl acetal.

The correctness of the experimental data of Pummerer *et al.*, as well as the deductions based thereon, have previously been questioned [2,3]. The experiments presented here are on the whole consistent with the main findings of Pummerer *et al.* (*cf.* also [7]), but their claim to have identified 5-methoxylevulinaldehyde, as well as all deductions based on this claim [5-8], cannot be accepted.

EXPERIMENTAL

Microanalyses by Ernst Boss and Kirsten Glens

Reaction of furfuryl alcohol with methanolic hydrogen choride (*cf.*[5,6]). Furfuryl alcohol (161 g, 1.64 mole), methanol (dried with magnesium, 1840 ml) and acetyl chloride (17.0 ml) were mixed and heated under reflux (3 h) [6]. After cooling a solution of sodium methoxide [from sodium (5.70 g) and methanol (70 ml)] was added and the mixture brought to pH 7 (test with wet indicator paper) by addition of small amounts of glacial acetic acid or sodium methoxide. The methanol was evaporated in a vacuum from a water-bath, a precipitate removed by filtration and washed with ether, and the filtrate distilled. The yield was 110.8 g of a slightly yellow liquid ($b.p._{0.3}$ 43—75°).

110.3 g of this product was fractionally distilled through a 25 cm packed column [9] into 26 fractions of approximately the same size (distillation pressure 21 mm, heat input 3.7 watt, take-off every 6.9 minutes, change of fraction every 8 hours, total distillation time 9 days) (Fig. 1). The refractive index and the infrared absorption of all fractions were measured. The yield of distillate was 102.7 g (93 % of distilland).

(a) The distillate boiling at about 88° (n_D^{25} 1.4235) (2.48 g) was heated under reflux (40 min) with sodium hydroxide (8 %, 10 ml) and the reaction mixture continuously extracted with ether. Distillation of the etheral extract gave 1.61 g of 2,3(or 4),5-trimethoxytetrahydrosilvan (I) (colorless liquid, $b.p._{10}$ 75°, n_D^{25} 1.4239). The compound showed no absorption band around 5.8 mμ. (Found: C 54.7; H 9.1; OCH_3 51.6. Calc. for $C_5H_7O(OCH_3)_3$ (176.2): C 54.5; H 9.2; OCH_3 52.8).

I (0.700 g, 0.0040 mole) was heated under reflux (10 min) with 0.025 *N* hydrochloric acid (4.0 ml). After cooling, the light-yellow reaction mixture was added to a solution of acetonedicarboxylic acid (1.80 g), methylamine hydrochloride (0.84 g), citric acid (3.00 g) and sodium hydroxide (2.36 g) in water (68 ml). The resulting solution (pH 5.0) was left standing (48 h) at room temperature. Potassium carbonate (anhydrous, 35 g) was added and dissolved, and the solution continuously extracted with ether. The ether was removed from the etheral extract by distillation. The oily residue was dissolved in ethanol (5 ml) and added to a solution of picric acid (1.20 g) in hot ethanol (10 ml). Hereby 0.570 g (35 %) of methylmethoxytropinone picrate was obtained [m. p. 129—131° (Hershberg

apparatus, corr.)]. Crystallization from ethanol gave 0.374 g (m. p. 135—136°; further crystallization did not change the m. p.). (Found: C 46.8; H 5.0; N 13.1; OCH_3 6.6. Calc. for $C_{15}H_{17}O_8N_4$ (OCH_3) (412.4): C 46.6; H 4.9; N 13.6; OCH_3 7.5).

The picrate was further characterized by its infrared spectrum (in potassium bromide), which was found to be different from the spectrum of 1-methoxymethyltropinone picrate (m. p. 164—170°), prepared from 2,5-dimethoxytetrahydrofurfuryl methyl ether [10].

The aqueous layer from the etheral extraction of trimethoxytetrahydrosilvan was acidified to Congo red with concentrated hydrochloric acid and extracted further with ether. The ether was removed by distillation and the oily residue dissolved in water (5 ml). Semicarbazide hydrochloride (0.5 g) and sodium acetate (0.75 g) were added and dissolved. After standing for 30 minutes a precipitate of levulinic acid semicarbazone was removed by filtration. The yield was 361 mg [m. p. 187° (dec.) (Hershberg apparatus, corr.)]. Crystallization from ethanol gave 296 mg [m. p. 187° (dec.)]. (Found: C 41.5; H 6.3; N 24.6. Calc. for $C_6H_{11}O_3N_3$ (173.2): C 41.6; H 6.4; N 24.3).

From the above data, analyses and infrared spectra, it is inferred that the distillate boiling at 88° is an azeotrope of 80 % of trimethoxytetrahydrosilvan and 20 % of methyl levulinate. In the separation experiment the yield of trimethoxytetrahydrosilvan (1.61 g) thus corresponds to 81 % and of levulinic acid semicarbazone (361 mg) to 55 %. (Found: C 55.1; H 8.8; OCH_3 47.5. Calc. for 80 % $C_5H_7O(OCH_3)_3$ + 20 % $C_5H_7O_2$ (OCH_3): C 54.7; H 8.9; OCH_3 47.0).

From the distillation curve (Fig. 1) the yield of trimethoxytetrahydrosilvan is estimated to 8.6 g (3.0 % based upon furfuryl alcohol) and of methyl levulinate to 2.2 g.

(b). The distillate boiling at about 94° (n_D^{25} 1.4201, n_D^{20} 1.4223, d_4^{20} 1.0490) is methyl levulinate, which previously has been prepared pure by Langlois and Wolff [11] (b. $p._{15}$ 89—91°, n_D^{20} 1.4225, d_4^{20} 1.0495). (Found: C 55.4; H 7.9; OCH_3 24.0. Calc. for $C_5H_7O_2$ (OCH_3) (130.1): C 55.4; H 7.8; OCH_3 23.8).

The yield of methyl levulinate is 57.0 g. This brings the total yield of methyl levulinate up to 59.2 g (27.7 %).

(c). The distillate boiling at about 112° (n_D^{25} 1.4229) is *a*(or *β*)-methoxylevulinaldehyde dimethyl acetal (III) [yield 29.2 g (10.1 %)]. (Found: C 54.8; H 9.2; OCH_3 52.6. Calc. for $C_5H_7O(OCH_3)_3$ (176.2): C 54.5; H 9.2; OCH_3 52.8).

III (2.56 g) was shaken (20 h) with a solution of sodium hydroxide (8 %, 50 ml) and the reaction mixture continuously extracted with ether and distilled under 11 mm. The distilland was dark-red and only 0.3 g of distillate, b. p. 115—162°, could be collected. III is thus transformed into undistillable material under the influence of alkali.

III (0.700 g, 0.040 mole) was hydrolyzed and the hydrolysate treated with acetonedicarboxylic acid and methylamine as described above. The yield of methylmethoxytropinone picrate was 0.504 g (31 %) (m. p. 135—136°). (Found: C 46.5; H 4.6; N 13.5; OCH_3 6.6).

The infrared spectrum was identical with that of the picrate of the tropinone from trimethoxytetrahydrosilvan.

(d). The distillate boiling between 94° and 112° contains

(1) a product (yield about 0.5 g), which has a larger refractive index than either methyl levulinate or methoxylevulinaldehyde dimethyl acetal. Of the fractions collected by us, the largest index (1.4258) was shown by the fraction (3.88 g) boiling from 103.1° to 108.2°. The product was not identified.

(2) Methyl levulinate dimethyl ketal [yield about 3.6 g (1.2 %)], which was identified by its spectrum. The fraction boiling from 103.1° to 108.2° was richest on ketal.

(3) A compound which turns yellow on standing. The compound, which is present in the fractions boiling above 103°, and in particular in the fraction boiling from 103.1° to 108.2°, was not identified. The yield of the compound is about 0.3 g. This brings the total yield of unknown compounds up to 0.8 g. Assuming an average molecular weight of 175, this corresponds to 0.3 %.

The yield of all distillable products from furfuryl alcohol is thus 42.3 %.

Reaction of furfuryl methyl ether with methanolic hydrogen chloride. Furfuryl methyl ether (184.0 g, 1.64 mole) was treated as described for furfuryl alcohol. The yield of distillable product was 101.9 g ($b.p._{0.5}$ 57—79°), 100.9 g of which was fractionated (Fig. 1) into 24 fractions. During the later half of the distillation methanol was conti-

nuously split off from the distilland. This was indicated by the accumulation of methanol in the cooling trap (3.8 g in all, n_D^{25} 1.3327). The yield of distillate, including the methanol, was 96.7 g (96 % of distilland).

(a). The distillate boiling at about 88° is identical with the 88° – distillate from furfuryl alcohol (inferred from the spectrum). The yield of trimethoxytetrahydrosilvan is 5.2 g (1.8 % based upon furfuryl methyl ether) and of methyl levulinate 1.3 g.

(b). The distillate boiling at about 94° (n_D^{25} 1.4218) is, as inferred from the spectrum and from analyses, an azeotrope of about 90 % of methyl levulinate and about 10 % of an unknown product, which has a considerably higher refractive index and a high content of methoxy groups. The yield of methyl levulinate is about 22.5 g and of the unknown product about 2.5 g. This brings the total yield of methyl levulinate up to 23.8 g (11.2 %). (Found: C 56.2; H 8.2; OCH_3 26.2).

(c). The distillate boiling between 88° and 94° and in particular the fraction (4.35 g) boiling from 89.8° to 93.2°, turns yellow on standing. The compound responsible for the coloration was not identified (yield about 0.5 g), but is probably identical with the above mentioned compound from furfuryl alcohol having the same property.

(d). The distillate boiling at about 103° (n_D^{25} 1.4239) is methyl levulinate dimethyl ketal (II) [yield about 44.3 g (15.3 %)]. The compound showed no absorption band around 5.8 mμ. (Found: C 54.9; H 9.4; OCH_3 51.6. Calc. for $C_5H_7O(OCH_3)_3$ (176.2): C 54.5; H 9.2; OCH_3 52.8).

II (3.00 g) was heated under reflux (3 h) with methanol (dried with magnesium, 3.0 ml) to which had been added water (310 mg) and concentrated hydrochloric acid (50 mg). Sodium (12 mg) was added and the mixture distilled. Hereby 1.90 g (86 %) of almost pure methyl levulinate was obtained (b. $p._{13}$ 83–86°, n_D^{25} 1.4208). (Found: C 55.6; H 8.6; OCH_3 25.5. Calc. for $C_7H_7O_3(OCH_3)$ (130.1): C 55.4; H 7.8; OCH_3 23.8).

II (3.80 g) was dissolved in a solution of methanol (10.0 ml), water (0.50 ml) and ammonia (2.3 g) and the resulting light-yellow solution left standing for 3 days. The solution was evaporated in a vacuum from a water-bath and the oily residue crystallized from benzene-petroleum ether. The resulting light-brown crystals were washed twice with benzene-petroleum ether and dried under 0.1 mm over sulfuric acid (3 h). The yield of impure levulinamide dimethyl ketal (VII) was 1.41 g (41 %) (m. p. 64–68°). Recrystallization from benzene-petroleum ether gave 1.10 g (32 %) of pure VII (slightly yellow crystals, m. p. 67–70°; further crystallization did not change the m. p.). (Found: C 52.1; H 9.5; N 9.1; OCH_3 38.0. Calc. for $C_5H_9ON(OCH_3)_2$ (161.2): C 52.2; H 9.4; N 8.7; OCH_3 38.5).

As explained in the following, only the intermediate fractions of the distillate boiling at about 103° are pure methyl levulinate ketal, while the first and the last fractions are contaminated with other compounds.

(e). The distillate boiling between 94° and 103° contains a product (yield about 1.4 g), which has a larger refractive index than either the 94°-azeotrope or methyl levulinate dimethyl ketal. Of the fractions collected by us, the largest index (1.4289) was shown by the fraction (3.88 g) boiling from 98.2° to 101.3°. The product which contaminates the first fractions of methyl levulinate dimethyl ketal was not identified, but is possibly identical with the above mentioned product from furfuryl alcohol having the same property.

(f). The distillate boiling above 103°. In this range at least one compound, which was not identified, was shown to be present as judged by the spectra. The compound contaminates the later fractions of the ketal. The data for the last 8 fractions of the distillation are given in Table 3.

The yield of unknown compounds boiling above 103° is about 15.0 g. This brings the total yield of unknown compounds up to 19.4 g. Assuming an average molecular weight of 190, this corresponds to 6.2 %.

The yield of all distillable compounds from furfuryl methyl ether is thus 34.5 %.

Reaction of dimethoxydihydrosilvan with methanolic hydrogen chloride. Dimethoxydihydrosilvan (237.0 g, 1.64 mole) was treated as described for furfuryl alcohol. The yield of distillable product was 209.0 g (b. $p._{0.6}$ 53–74°), 121.8 g of which was fractionated into 32 fractions (Fig. 1). Methanol was split off from the distilland as in the furfuryl methyl

Table 3. Data for the last 8 fractions of the distillation of the reaction product from furfuryl methyl ether.

Fraction (g)	B. p. °C	n_D^{25}	C	H	OCH_3
17 (3.69)	103.7	1.4239			
18 (3.73)	103.7—103.8	1.4239	55.7	9.4	51.1
19 (3.56)	103.8—104.2	1.4241			
20 (3.74)	104.2—104.8	1.4252	55.6	9.3	53.4
21 (3.92)	104.8—107.2	1.4278	55.6	9.3	56.7
22 (3.65)	107.2—108.4	1.4301	56.4	9.3	57.3
23 (3.80)	108.4—109.0	1.4291			56.4
24 (3.97)	109.0—109.8	1.4267			

ether experiment (yield 3.5 g, n_D^{25} 1.3397). The yield of distillate, including the methanol, was 115.4 g (95 % of distilland).

(a). The distillate boiling at about 87° is identical with the 88°-distillate from furfuryl alcohol. The yield of trimethoxytetrahydrosilvan is 4.7 g (2.8 % based upon dimethoxydihydrosilvan) and of methyl levulinate 1.2 g.

(b). The distillate boiling at about 93° (n_D^{25} 1.4212) is identical with the 94°-distillate from furfuryl methyl ether. The yield of methyl levulinate is about 17.7 g and of the unknown product about 2.0 g. This brings the total yield of methyl levulinate up to 18.9 g (15.2 %). (Found: C 55.7; H 8.1; OCH_3 26.3).

(c). The distillate boiling at about 102° (n_D^{25} 1.4229) is methyl levulinate dimethyl ketal. The yield is about 56.9 g (33.8 %). (Found: C 54.5; H 9.4; OCH_3 52.6).

(d). The distillate boiling between 93° and 102° contains a product (yield about 1.2 g), which has a larger refractive index than either the 93°-azeotrope or methyl levulinate dimethyl ketal. Of the fractions collected by us, the largest index (1.4249) was shown by the fraction (4.1 g) boiling from 101.0° to 101.6°. The product, which contaminates the first fractions of methyl levulinate dimethyl ketal, was not identified, but is possibly identical with the above mentioned product from furfuryl alcohol and furfuryl methyl ether having the same property.

(e). The distillate boiling above 102°. In this range at least 2 different compounds, which were not identified, were shown to be present as judged by the spectra. The lowest boiling of these, which is identical with the unknown compound boiling in the same range in the furfuryl methyl ether experiment, contaminates the later fractions of methyl levulinate dimethyl ketal. The data for the last 12 fractions of the distillation are given in Table 4.

The yield of unknown compounds boiling above 102° is about 28.0 g. This brings the total yield of unknown compounds up to 31.2 g. Assuming an average molecular weight of 190, this corresponds to 17.3 %.

The yield of all distillable products from dimethoxydihydrosilvan is thus 69.1 %.

Treatment of the reaction mixture from furfuryl methyl ether with methanolic potassium hydroxide. Furfuryl methyl ether (350.0 g) was heated under reflux with methanolic hydrogen chloride as described above. Potassium hydroxide pellets (250 g) were added and the mixture heated further under reflux (18 h). The major part of the methanol was removed from the black reaction mixture by distillation in a vacuum. Water (150 ml) was added and the remainder of the methanol was removed by distillation under 200 mm through a Vigreux type column. Another 150 ml of water was added and the mixture

Table 4. Data for the last 12 fractions of the distillation of the reaction product from dimethoxydihydrosilvan.

Fraction (g)	B. p. °C	n_D^{25}	C	H	OCH_3
21 (4.13)	102.2—102.3	1.4230			
22 (4.11)	102.3—102.7	1.4232	55.7	9.7	52.8
23 (4.11)	102.7—102.9	1.4238			52.8
24 (4.08)	102.9—104.0	1.4250			54.1
25 (4.07)	104.0—106.2	1.4280			57.1
26 (4.08)	106.2—107.2	1.4296			58.0
27 (4.07)	107.2	1.4292			57.0
28 (4.07)	107.2—107.5	1.4286	55.1	9.4	57.2
29 (4.06)	107.5—107.8	1.4272			56.1
30 (3.97)	107.8—112.1	1.4286			56.4
31 (3.09)	112.1—114.8	1.4400			57.8
32 (0.41)	114.8—116.1	1.4485	56.1	9.3	54.6

continuously extracted with ether in a Kutscher-Steudel apparatus for 2 days. The black liquid in the boiling flask (2 layers) was shaken with anhydrous magnesium sulfate (100 g), filtered, and the magnesium sulfate washed with ether. The combined etheral solutions (0.5 l) were shaken with a saturated solution of calcium chloride (0.5 l), dried with potassium carbonate and distilled under 0.2 mm. The yield of distillate was 25.3 g (colorless liquid, b. p. 65—71°, n_D^{25} 1.4254), a portion of which (23.2 g) was fractionated through a 12 cm column [9] into 12 fractions (distillation pressure 12 mm, heat input 3.6 watt, take-off every 2.4 minutes, change of fraction every hour, total distillation time 12 hours) (Fig. 2 and Table 5). The infrared absorption of all fractions was measured. The yield of distillate was 21.5 g (93 % of distilland).

(a). The distillate boiling at about 73° is the same trimethoxytetrahydrosilvan as isolated above (inferred from the spectrum).

(b). The distillate boiling at about 91° consists of a main product (inferred from the spectrum) and another product, which turns yellow on standing. Fractions 4 and 5 turn strongly yellow, and fractions 6—9 weekly yellow. None of the compounds were identified, but the compound responsible for the coloration is probably identical with the above mentioned compound from furfuryl alcohol and furfuryl methyl ether having the same property. Since the compound is alkali stable, and is not found in the reaction product from dimethoxydihydrosilvan, we believe it to be a furan compound, which is autoxidized on standing.

(c). The distillate boiling at 104° is 1,1,2(or 3),4,4-pentamethoxypentane (VIII). [Calc. for $C_5H_7(OCH_3)_5$ (222.3): C 54.0; H 10.0; OCH_3 69.8]. The compound showed no absorption band around 5.8 mμ.

0.900 g of pentamethoxypentane (0.040 mole) was hydrolyzed and the hydrolysate treated with acetonedicarboxylic acid and methylamine hydrochloride as described above. The yield of methylmethoxytropinone picrate was 0.600 g (36 %) (m. p. 132—136°). Crystallization from ethanol gave 0.500 g (m. p. 133—134°). (Found: C 46.6; H 4.6; N 13.6; OCH_3 7.2).

Table 5. Data for the distillation of alkali-resistent compounds from furfuryl methyl ether.

Fraction (g)	B. p. °C	n_D^{25}	C	H	OCH_3
1 (1.35)	70.5— 73.0	1.4242	55.4	9.3	52.4
2 (1.52)	73.0— 73.6	1.4236	55.0	9.6	51.5
3 (1.97)	73.6— 76.0	1.4239	54.9	9.4	51.5
4 (2.02)	76.0— 88.5	1.4256	54.8	10.1	54.4
5 (2.02)	88.5— 90.3	1.4268	55.2	9.5	53.8
6 (2.06)	90.3— 91.0	1.4262	55.1	9.6	55.3
7 (2.04)	91.0— 91.5	1.4261	55.4	9.6	53.5
8 (1.99)	91.5— 92.4	1.4258	55.1	9.4	53.7
9 (1.98)	92.4— 98.6	1.4258	55.3	9.5	56.6
10 (1.89)	98.6—103.2	1.4243	54.8	9.9	65.4
11 (1.71)	103.2—104.0	1.4232	54.2	9.8	70.6
12 (0.91)	104.0	1.4231	54.6	10.4	68.5

The infrared spectrum of the picrate was identical with that of the picrate of the tropinone from trimethoxytetrahydrosilvan.

The yield of trimethoxytetrahydrosilvan, as estimated by the distillation curve, is 5.5 g (1.1 %), of pentamethoxypentane 4.7 g (0.7 %) and of unknown compounds 11.1 g (about 2.5 %).

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Studies on Carbamates

XI. The Carbamate of Ethylenediamine

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The velocity constants of the reaction $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2 + CO_2 \rightleftharpoons {}^+H_3N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$ and the equilibrium constant for the reaction $H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^- + H_2O \rightleftharpoons H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2 + HCO_3^-$ have been determined. The velocity of the decomposition of $H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$ in basic medium was investigated and may be explained in assuming that the decomposition is a two stage reaction, *viz.* 1) carbamate $\rightleftharpoons$ amine + carbon dioxide, 2) carbon dioxide $\rightleftharpoons$ carbonate.

It is to be noticed that the carbamate has been prepared not only in solution by leading CO_2 into a diluted solution of $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$ but also as a solid. This substance consists of equal moles of $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$ and CO_2 so that the gross composition corresponds with both monocarbamate, $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$, and dicarbamate, $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NH_2^+$, $^-OOCHN \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$. The carbamate showed by analysis that it did not contain monocarbamate exclusively, approx. 20 % CO_2 being present as dicarbamate. The experiments on equilibrium and velocity have been calculated as if all the carbamate was present as monocarbamate.

1. The equilibrium conditions and reaction mechanism of the formation and decomposition in aqueous medium of the carbamate formed by ethylenediamine have been studied. As the experimental and theoretical conditions are practically analogous to those of the carbamates previously investigated, we confine ourselves, with some exceptions, to refer to an earlier paper [1,2] for the detailed information concerning method, theory, significance of constants, *etc.* It should be noted, though, that "Am" means $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$ and "AmH^+" $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NH_2$.

2. Two different preparations of ethylenediamine were used in the experiments. One preparation was purified through the acid oxalate by recrystallization, until constant molecular weight was obtained. The molecular weight of the acid oxalate, crystallizing with one molecule of crystal water, is theoretically 258.20. The preparation obtained showed the following molecular weight: By titration with permanganate it was found to be 256.8, and by a Kjeldahl

analysis it was found to be 262.1; the melting point was about 210°C. From the acid oxalate was prepared an aqueous solution of ethylenediamine by distilling it off with sodium hydroxide.

The other preparation was purified by distillation in an ordinary distiller at atmospheric pressure after drying with metallic sodium; the fraction used had a boiling point of 117.3—117.7° C (768 mm Hg), $n_D^{25.0°} = 1.4548$, and by titration with 1.0 *N* HCl a molecular weight of 61.7 (theoretically 60.1) was found. Presumably this difference in the molecular weight is due to small contents of water.

The two preparations gave the same results in the experiments concerning the formation and decomposition of carbamate.

3. The carbamate of ethylenediamine was prepared partly in solution by leading a deficit of carbon dioxide to aqueous solutions of the amine, practically all of the carbon dioxide thus being converted to carbamate, and partly as a solid.

The solid substance, the gross composition of which corresponds with equal moles of $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$ and CO_2 was prepared in the following way. Carbon dioxide was lead through a mixture of 80 g amine and 20 g water, cooled to 0° C, for an hour; after ten hours at 0° C a microcrystalline precipitate was formed, which increased for the following fifty hours at 20° C. It was dried and stored over concentrated sulphuric acid in an exsiccator, in which it, at least for some time, showed no sign of decomposition. 58.0 % of amine (theoretically 57.73 %) was found by titration of the carbamate with 0.1 *N* HCl, and by the method of analysis, stated under 4, 42.0 % of carbon dioxide (theoretically 42.27 %) in the form of carbamate was found.

The gross composition corresponds with two compounds, *viz.* $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$ and $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NH_3^+$,$^-OOCHN \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$. Katchalski, Berliner-Klibanski and Berger [3-5] have recently shown that a carbamate they prepared as a solid substance was a mixture of approx. equal parts by weight of monocarbamate and dicarbamate.

We have examined the same problem but after quite another method than the one used by the above-mentioned authors. Our principle has been to dissolve the preparation of carbamate in excess of 1.0 *N* NaOH. By this method 1 mole of amine was liberated from 1 mole of dicarbamate but no amine was liberated from monocarbamate. The solution was immediately shaken with *n*-amyl alcohol whereupon the concentration of $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$ in the layer of *n*-amyl alcohol was determined by titration. Approximately, the partition coefficient of $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$ between *n*-amyl alcohol and 1.0 *N* NaOH was found equal to 0.10. By means of the titration results and the partition coefficient the concentration of liberated amine was calculated. The indicator was bromophenol blue, and the layer of *n*-amyl alcohol was dissolved in enough *iso*-propyl alcohol to ensure homogeneity after titration. The examination showed that approx. 20 % CO_2 was present as a dicarbamate, *i.e.* approx. 80 % CO_2 was present as a monocarbamate.

4. The method of analysis was as in previous investigations precipitation with barium chloride, causing the precipitation of carbonate, but not of carbamate. All of the data presented in the later tables are corrected for blank values, *viz.* 3—5 units of the percentage.

5. All of the experiments were performed at 18° C, and the velocity constants were calculated by means of Briggs' logarithms, the unit of time being the minute. As in previous investigations the activity coefficient f for a monovalent ion was calculated from the expression of Bjerrum $-\log f = 0.3 \sqrt[3]{c_{ion}}$.

6. For the calculation of certain experiments we have needed the value of the equilibrium constant of $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_3^+ \rightleftharpoons H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2 + H^+$. Since the values of K_{AmH^+} stated in the literature [6-8] not agree very well, we have used a value determined by us. This constant was calculated (Table 1) on the basis of determinations of the hydrogen ion activity in solutions containing equal moles of corresponding acid and base, prepared by the mixing of ethylene diamine with half the number of moles of hydrochloric acid. The investigations were carried out both by means of a hydrogen electrode against a 0.1 N calomel electrode and by means of a glass electrode. By the hydrogen electrode the hydrogen ion activity was calculated from the expression $pa_H = -\log a_H{}^+ = (E - E_o) \frac{F \log e}{RT}$, E_o being fixed at 0.3360 [9], and the adjustment of the glass electrode was carried out by means of buffer solutions of phosphate, borate and sodium hydroxide, according to Sørensen. No corrections were made for the diffusion potentials, these being insignificant.

Table 1. Determination of the second acidic dissociation constant of ethylenediamine 18°.

c_{acid}	c_{base}	pa_H	
		Glass electrode	Hydrogen electrode
0.198	0.198	10.19	10.22
0.148	0.148	10.17	10.21
0.099	0.099	10.16	10.19
0.079	0.079	10.14	10.18
0.060	0.060	10.21	10.18
0.049	0.049	10.13	10.16
0.039	0.039	10.19	10.17

The results are found in Table 1, from which it appears that the expression

$$\frac{a_H{}^+ \times c_{H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2}}{c_{H_3N \cdot CH_2 \cdot CH_2 \cdot NH_2{}^+}} = K'_{AmH^+}$$

has a practically constant value independent of the ion concentration. We have used $K_{AmH^+} = 10^{-10.17}$ in the calculations.

The acidic dissociation constant for $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NH_3^+ \rightleftharpoons H_2N \cdot CH_2 \cdot CH_2 \cdot NH_3^+ + H^+$ was calculated by us on the basis of similar determinations as mentioned before and was found to be about $10^{-7.2}$. In the calculations of our experiments we have only used K'_{AmH^+}, since the solutions are so basic, that they contain practically nothing of the ion $^+H_3N \cdot CH_2 \cdot CH_2 \cdot NH_3^+$.

7. The acidic dissociation constant

$$K'_{amate} = \frac{c_{H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-} \times a_H{}^+}{c^+_{H_3N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-}}$$

for the process: $^{+}H_3N \cdot CH_2 \cdot CH_2 \cdot NHCOO^- \rightleftharpoons H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^- + H^+$ has been determined in the following way. By dissolving the carbamate, $^{+}H_3N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-$, as quickly as possible in a deficit of sodium hydroxide solution, we made solutions containing acid and corresponding base, and in these solutions we determined pa_H by means of a glass electrode in the course of a few minutes. It should be noted, that we, during this short time, took the liberty of ignoring the decomposition of the carbamate. The results are found in Table 2, from which it appears, that

$$K'_{amate} = \frac{c_{H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-} \times a_{H^+}}{c^+_{H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-}}$$

is independent of the ion concentration analogous to the conditions of the alanines [1].

Table 2. Determination of the acidic dissociation constant of the carbamate 18°.[1]

c_{acid}	c_{base}	pa_H	pK'_{amate}
0.0025	0.0025	10.09	10.09
0.005	0.005	10.10	10.10
0.0125	0.0375	10.58	10.10
0.025	0.025	10.10	10.10

i. e. pK'_{amate}: 10.10

On the reaction "amine + carbon dioxide → carbamic acid"

500 ml of an aqueous solution containing both amine and sodium hydroxide were shaken vigorously for two minutes with a deficit of a gaseous mixture of 15 % carbon dioxide and 85 % atmospheric air. We also tried in the course of approx. 15 minutes to lead atmospheric air containing about 1 % carbon dioxide into the solution of both amine and sodium hydroxide, the decomposition of the carbamate going so slowly, that we can disregard it. The mixture was immediately analysed, and the two methods gave practically the same results. The analytical data obtained in the experiments are listed in Table 3, where "% carbamate" indicates how many per cent of the carbon

Table 3. Carbon dioxide in amine + NaOH, 18°.

Initial solution		Absorbed CO_2 $\frac{mole}{litre}$	% carbamate	Final solution		Mean		$k_{CO_2 \cdot Am}$	
c_{NaOH}	c_{Am}			c_{NaOH}	c_{Am}	c_{NaOH}	c_{Am}		Mean
0.20	0.10	0.0178	51	0.18	0.09	0.19	0.10	$10^{5.34}$	
0.20	0.10	0.0266	48	0.16	0.09	0.18	0.09	$10^{5.27}$	$10^{5.30}$
0.20	0.10	0.0116	50	0.18	0.10	0.19	0.10	$10^{5.30}$	

Table 4. The solution of carbonate-carbamate in equilibrium, 18°.

Initial solution				% carbamate	Equilibrium				K_{Eq}	
c_{Am}	c_{AmH^+}	$c_{(AmH)_2CO_3}$	$c_{carbamate}$		c_{Am}	c_{AmH^+}	$c_{carbamate}$	$c_{HCO_3^-}$		Mean
0.01	0.00	0.04		74 [1]	0.020	0.015	0.0089	0.0023	$10^{-2.29}$	$10^{-2.32}$
0.01	0.01		0.02	79 [2]	0.0057	0.0186	0.0042	0.0033	$10^{-2.34}$	

[1] Mean of 5 determinations: 73.1 73.7 73.3 74.3 74.1.
[2] » » 5 » 78.2 78.4 79.2 78.8 79.2.

dioxide absorbed have been converted to carbamate. Furthermore, the velocity constant $k_{CO_2 \cdot Am}$ for the reaction $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2 + CO_2 \rightarrow H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOOH$ was calculated.

The equilibrium "carbamate ⇌ carbonate"

Experiments with ethylenediamine have been done from the carbonate side as well as from the carbamate. In Table 4 are listed the compositions of the solutions and the equilibrium constant K_{Eq} for the reaction $H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^- + H_2O \rightleftharpoons H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2 + HCO_3^-$.

It should be noted that in the experiments

$c_{Am} = c_{H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2}$, $c_{AmH^+} = c_{H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2^+}$ and $c_{amate} = c_{H_2N \cdot CH_2 \cdot CH_2 \cdot NHCOO^-}$, c_{amate} being calculated on the basis of $c_{+H_2N \cdot (CH_2)_2 \cdot NHCOO^-}$, a_{H^+} and K'_{amate}.

Table 5. Velocity constants for the process "carbamate ⇌ carbonate"; pa_H = approx. 10—11; 18°.

Initial solution			Min.	% carbamate	$k_{amate} + k_{onate}$
	c_{AmH^+}	c_{Am}			
0.020 *M* $(AmH)_2CO_3$	0.05	0.11	40	5.5	0.000707
			100	13.3	0.000712
			150	18.2	0.000673
			210	24.7	0.000682
			325	36.5	0.000716
					Mean : 0.00070
					k_{amate} : 0.000085
					k_{onate} : 0.00061
0.020 *M* $(AmH)_2CO_3$	0.10	0.13	80	11.4	0.000711
			170	23.5	0.000745
			260	33.2	0.000740
			365	43.4	0.000748
			470	52.2	0.000762
					Mean : 0.00075
					k_{amate} : 0.000055
					k_{onate} : 0.00070

The velocity of the conversion "carbamate ⇌ carbonate"

In Table 5 are presented the experiments on velocity, which have been carried out in a buffer solution consisting of $^{+}H_3N \cdot CH_2 \cdot CH_2 \cdot NH_2$ / $H_2N \cdot CH_2 \cdot CH_2 \cdot NH_2$, where a measurable equilibrium is established between carbamate and carbonate. In Table 6 are presented those experiments which have been carried out in a medium containing sodium hydroxide, where carbamate is converted almost completely to carbonate.

The velocity constants calculated from the experiments are listed in Table 5 and 6. These velocity constants may be calculated in advance, provided the decomposition takes place through the reactions

$$\text{carbamate} \rightleftharpoons \text{amine} + CO_2$$
$$CO_2 \rightleftharpoons \text{carbonate}.$$

Table 6. Velocity constants for the process "carbamate → carbonate"; pa_H = approx. 13; 18°.

Initial solution			Min.	% carbamate left	k_{amate}
$c_{carbamate}$	c_{NaOH}	c_{Am}			
0.022 *M* (CO_2 in solution of amine)	0.08	0.08	0	100	
			180	96.2	0.0000917
			1494	73.1	0.0000913
			2892	54.4	0.0000909
			4295	42.1	0.0000876
			5764	31.2	0.0000878
			10057	12.6	0.0000894
				Mean:	0.000090
0.021 *M* (preparation of carbamate)	0.08	0.05	0	100	
			399	89.7	0.000118
			1426	69.3	0.000112
			2855	49.9	0.000106
			5742	25.4	0.000104
			7289	17.7	0.000103
			8819	13.0	0.000101
			10233	8.9	0.000103
				Mean:	0.000107

Table 7. Velocity constants, experimental and calculated.

Initial solution					k_{amate}		k_{onate}	
$c_{(AmH)_2CO_3}$	$c_{carbamate}$	c_{AmH^+}	c_{Am}	c_{NaOH}	exptl.	calc.	exptl.	calc.
0.02		0.05	0.11		0.000085	0.000087	0.00061	0.00066
0.02		0.10	0.13		0.000055	0.000076	0.00070	0.00079
	0.022		0.08	0.08	0.000090	0.000079		
	0.021		0.05	0.08	0.000107	0.000098		

In Table 7 is given a survey of the experimental and calculated values of the velocity constants.

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Studies on Local Anesthetics XI *

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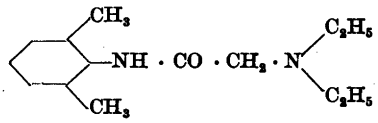
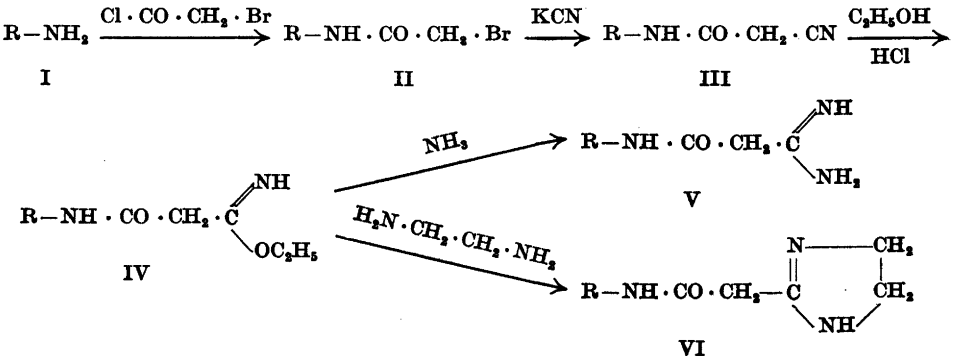
Three new compounds, related to xylocaine(α-diethylamino-2,6-dimethylacetanilide), *viz.* β-imino-β-ethoxy-2,6-dimethylpropionanilide, α-guanyl-2,6-dimethylacetanilide and α-(2-imidazolin-2-yl)-2,6-dimethylacetanilide have been synthesized and tested pharmacologically. In comparison with xylocaine the imido-ester and the imidazoline have very slight anesthetic activities on the rabbit cornea. The amidine is inactive. On the blood pressure the amidine and the imidazoline, like xylocaine, have a weak depressant action.

The syntheses of three compounds in which the diethylamino group of xylocaine



Xylocaine

is replaced by an imidocarbethoxy, a guanyl or a 2-imidazolin-2-yl group were carried out according to the following scheme:



R = 2,6-$(CH_3)_2C_6H_3$

* For paper X of this series see Löfgren and Tegnér [1].

The bromoacetylation of 2,6-dimethylaniline (I) yielding α-bromo-2,6-dimethylacetanilide (II) was carried out according to the aqueous acetate buffer method described by Löfgren [2,3]. Heating of II with KCN in ethanol gave α-cyano-2,6-dimethylacetanilide (III). This derivative was converted into β-imino-β-ethoxy-2,6-dimethylpropionanilide (IV) by reaction with ethanol and dry hydrogen chloride in dioxan solution, the procedure being a modification of that used by Cook and Reed [4] for the preparation of 1,4-bis(3-ethoxy-3-iminopropoxy)benzene. The ammonolysis of the imido-ester IV forming α-guanyl-2,6-dimethylacetanilide (V) was carried out in absolute ethanol during 5 days at 0°, and then for 2 days at room temperature. When condensing IV with ethylene diamine to give α-(2-imidazolin-2-yl)-2,6-dimethylacetanilide (VI) we followed the directions given by Klarer and Urech [5] who synthesized 2-(hydroxymethyl)-2-imidazoline by reaction between ethyl glycolimidate and ethylene diamine in absolute ethanol. To get a satisfactory yield of VI we had to perform the reaction at a lower temperature and over a longer period than is described in the synthesis of Klarer and Urech who obtained a high yield of their compound.

The compounds IV, V and VI were tested for their local anesthetic activity on the rabbit cornea and compared with xylocaine, the method given by Wiedling being followed [6]. In these experiments the imido-ester IV and the imidazoline VI gave very slight anesthetic activities. The amidine V was found to be inactive *.

In common with local anesthetics in general xylocaine exercises a weak hypotensive action [6,7]. The effect on blood pressure of IV, V, VI and xylocaine were compared in the rabbit **. It was found that the imido-ester IV was devoid of action whereas the amidine V and the imidazoline VI, like xylocaine, gave a weak depressant action according to the following order of magnitude: V > VI > xylocaine.

As shown previously by Wiedling [6] in experiments on isolated guinea pig intestine xylocaine is devoid of histaminolytic action but has a slight cholinolytic activity, *i.e.* about 1 % of that of diphenhydramine. For an estimation of the histaminolytic and cholinolytic effects of compounds IV, V and VI the above mentioned method of Wiedling was used. No appreciable effects were found.

By subcutaneous injections in white mice the toxicities were determined for V and VI. The toxicity of IV was not investigated since its solubility in acids was too low to allow a proper determination. The LD_{50} values of the amidine V and the imidazoline VI, calculated as grams of the base per kilogram of body weight were found to be 0.45 and 0.24, respectively. The corresponding value for xylocaine [6] is 0.34. In terms of *molar toxicity*, *i.e.* the toxicity expressed in moles per kilogram of body weight, the LD_{50} values of V, VI and xylocaine are $2.2 \cdot 10^{-3}$, $1.0 \cdot 10^{-3}$ and $1.4 \cdot 10^{-3}$, respectively.

* No action of 0.25 ml of a 2 % hydrochloride solution of pH 7.3 even after 4 minutes' exposure to the eye.

** Intravenous supply to amytal anesthetized rabbits.

EXPERIMENTAL *

α-Bromo-2,6-dimethylacetanilide (II). This compound was prepared by reaction between 2,6-dimethylaniline and bromoacetyl chloride in an acetate buffer, the general directions of Löfgren [2,3] for this type of reaction being followed. The yield of the crude α-bromo-2,6-dimethylacetanilide melting at 145—150° was 91 %. On recrystallization from absolute ethanol the compound was obtained as colourless needles. M. p. 155—156° (corr.). (Found: C 49.7; H 4.99. Calc. for $C_{10}H_{12}BrNO$ (242.1): C 49.6; H 5.00).

α-Cyano-2,6-dimethylacetanilide (III). In a round-bottomed flask equipped with a stirrer and a reflux condenser a solution of 20 g (0.083 mole) of α-bromo-2,6-dimethylacetanilide in 140 ml of absolute ethanol was heated to 60°. To the hot solution were added in small portions and during a period of 45 minutes 7.2 g (0.11 mole) of potassium cyanide, dissolved in 9 ml of water. On the addition a precipitate formed. The mixture was then diluted with 20 ml of absolute ethanol and under continued stirring the temperature was maintained at 60° for another 2 hours. Finally, the mixture was heated to 80° and filtered by suction through a pre-heated glass filter. After dilution with a large quantity of water the precipitate was filtered off, washed with water and dried in a vacuum desiccator over silica gel. On recrystallization from 96 % ethanol pale yellow needles were obtained. M. p. 210—212° (corr.); yield 6.4 g (0.034 mole), *i. e.* 41 %. (Found: C 70.4; H 6.43. Calc. for $C_{11}H_{12}N_2O$ (188.2): C 70.2; H 6.43.)

β-Imino-β-ethoxy-2,6-dimethylpropionanilide (IV). A mixture of 5.1 g (0.027 mole) of finely ground α-cyano-2,6-dimethylacetanilide, 1.3 g (0.027 mole) of absolute ethanol and 60 ml of highly purified dioxan was placed in a 250-ml round-bottomed flask fitted with a gas inlet tube, a thermometer and an ascending reflux condenser with a calcium chloride tube. Through the gas inlet tube a moderate stream of hydrogen chloride, carefully dried by passage through a mixture of phosphorus pentoxide and asbestos, was introduced. The temperature of the reaction mixture was not allowed to rise above 40°. After 4—5 hours the solution was cooled to 5° and the introduction of gas was continued until saturation. The reaction vessel was then closed and kept at a temperature of 4—5° for two days. After that time the expected *hydrochloride* of the imido-ester had partly precipitated. The precipitation was completed by dilution with absolute ether. The substance was filtered off, washed with absolute ether and dried in a vacuum desiccator over potassium hydroxide and silica gel. M. p. 128—129°; yield 6.7 g (0.025 mole), *i. e.* 91 %. (Found: Cl 13.16 (Mohr). Calc. for $C_{13}H_{19}ClN_2O_2$ (270.8): Cl 13.10.)

In some runs a less pure product was obtained since a small quantity of ammonium chloride contaminated the imido-ester hydrochloride. Satisfactory purification could be obtained by dissolving the substance in cold ethanol followed by fractional precipitation with dry ether. However, a more convenient purification was obtained by preparing the free base followed by recrystallization. For this purpose the salt preparation was dissolved in cold water and the imido-ester base liberated by the addition of dilute ammonia. The base was taken up in ether and after washing with water and drying with sodium sulphate the ether was driven off, leaving the imido-ester as needles. The base was dissolved at room temperature in a little more than the required amount of xylene (commercial mixture of isomers) and after addition of some light petroleum the compound was recrystallized by cooling to about −10°. M. p. 87—89°. (Found: C 66.9; H 7.48. Calc. for $C_{13}H_{18}N_2O_2$ (234.4): C 66.6; H 7.74.)

α-Guanyl-2,6-dimethylacetanilide hydrochloride (V). An alcoholic ammonia solution was prepared by saturating 10 ml of absolute ethanol at 0° with dry ammonia gas and diluting with 10 ml of alcohol. To this solution, 5.0 g (0.018 mole) of β-imino-β-ethoxy-2,6-dimethylpropionanilide · HCl were added. The mixture which was kept in a glass stoppered bottle was well shaken and allowed to stand for 5 days at 0° and for another 2 days at room temperature. After that time the mixture was filtered to remove the ammonium chloride which had precipitated. The red-violet filtrate was diluted with absolute ether until no more precipitate was formed. The precipitate was filtered off and washed with dry ether. This product in the form of a fine white crystalline powder was the desired amidine hydrochloride and did not need any further purification. M. p.

* All melting points are uncorrected unless otherwise stated.

230°; yield 3.1 g (0.013 mole), *i. e.* 72 %. (Found: C 54.4; H 6.63; Cl 14.93 (Mohr). Calc. for $C_{11}H_{16}ClN_3O$ (241.7): C 54.7; H 6.76; Cl 14.67).

α-(2-Imidazolin-2-yl)-2,6-dimethylacetanilide hydrochloride (VI). A solution of 1.1 g (0.019 mole) of ethylene diamine in 15 ml absolute ethanol was placed in a 100-ml two-necked flat-bottomed flask equipped with a magnetic stirrer and an ascending reflux condenser with a drierite tube. The solution was cooled to −15° and 5.2 g (0.019 mole) of β-imino-β-ethoxy-2,6-dimethylpropionanilide · HCl were added under vigorous stirring. The mixture was kept at −15° for half an hour and then at 0° for two hours after which time 11 ml of 1.74 *N* alcoholic hydrogen chloride (0.019 mole of HCl) were added under continued stirring. The flask was now allowed to assume room temperature and to remain at that temperature for 2 hours. The mixture was then heated at 40° for 2 hours, stirring being continued. After that time the mixture was filtered by suction through a pre-heated glass filter. On cooling, the hydrochloride of α-(2-imidazolin-2-yl)-2,6-dimethylacetanilide separated as colourless crystals. They were collected and washed with dry ether. The product did not need further purification. M. p. 238−240°; yield 2.7 g (0.010 mole), *i. e.* 53 %. (Found: C 57.9; H 6.70. Calc. for $C_{13}H_{18}ClN_3O$ (267.8): C 58.3; H 6.77.)

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Studies on Methods for the Determination of Lipoperoxides

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1. Various improvements and modifications of the colorimetric indophenol method for peroxide determinations are described. The method can be carried out as a "cold" method when a small amount of ferric chloride is added. The extinction produced by 0.001 milliequivalent of peroxide in 5 ml volume using benzene-ethanol or chloroform-ethanol as a solvent is about 1.00 at 520 mμ.

2. The bleaching effect of phospholipids in the thiocyanate method for peroxide determination is caused by the complex-binding of iron to the phosphoric acid groups of the phospholipids. It can be overcome by increasing the thiocyanate concentration.

3. Methods for the determination of peroxide groups in phospholipids and triglycerides containing high amounts of phospholipids are described.

4. The iodometric, the indophenol, and the thiocyanate methods for peroxide determination were compared. The latter method, carried out in the absence of oxygen, was found to be most suitable.

In a previous paper from this laboratory a new method, the colorimetric indophenol method, for the determination of the peroxides of fats and fatty acids (lipoperoxides) was described [1]. The method consists of heating the lipoperoxide-containing fat in a xylene-acetic acid solution with dichlorodihydroxydiphenylamine, the leuco-compound corresponding to the well-known redox indicator dichlorophenolindophenol. By oxidation of the leuco-compound, an amount of the indophenol equivalent to the quantity of lipoperoxide is formed. From the intensity of the red color of the indophenol the peroxide content is calculated.

The method has been used in this laboratory for several years and has been found sensitive and reproducible for the determination of lipoperoxides in several kinds of material. In the course of this work a number of improvements have been introduced in the method. The first part of this paper deals with our experiences with the colorimetric indophenol method, and at the same time two publications from other investigators [2,3], who have criticized the method, are discussed. Later parts of the paper contain a comparison of our method with other methods for the determination of peroxides as well as a study of the influence of phospholipids on the determination of peroxides.

A. THE COLORIMETRIC INDOPHENOL METHOD

Purification of solvents. All solvents used in work with lipoperoxides must be of a high degree of purity and free of peróxides as well as reducing substances and of heavy metal contamination. Peroxide-containing solvents can be purified by distillation under suitable conditions, for instance, water vapor distillation (toluene), or by filtering through a column of alumina (petroleum ether, benzene, toluene).

Several solvents (*e. g.*, petroleum ether and xylene) only remain peroxide-free for a very short time after they have been purified. On the other hand, benzene, chloroform, methanol and ethanol remain peroxide-free almost indefinitely, and good commercial qualities can be used without purification.

Preparation of color reagent. Dichlorodihydroxydiphenylamine, the leuco compound corresponding to dichlorophenolindophenol, is prepared by reduction of the latter by the following simplified method.

Two grams of 2,6-dichlorophenolindophenol (the product from British Drug House Company was found to be of a superior quality) are dissolved in 200 ml of hot distilled water. After filtration, the dye is reduced by the addition of about 1 g ascorbic acid. The precipitate of dichlorodihydroxydiphenylamine (in the following abbreviated "leuco") is filtered off on a glass filter and washed with redistilled water. When dissolved in a small quantity of absolute ethanol, some dark colored impurities are left on the filter. Further quantities of brownish impurities are removed by shaking the alcoholic solution with a small amount of activated carbon, followed by filtration. The leuco is precipitated again by the addition of redistilled water, filtered off, washed with redistilled water, and stored *in vacuo* over silica gel desiccant.

The leuco forms needles that are snow-white when freshly prepared but assume a bluish metallic appearance due to oxidation when stored for a long time. The use of redistilled water is essential, since the presence of small amounts of heavy metals generally occurring in ordinary distilled water markedly reduces the stability of leuco, especially in solution.

The color reagent is a 2 % solution of leuco in absolute ethanol. It keeps for several days when stored in a brown bottle, preferably in the ice-box. Two drops of the solution taken with a dropping pipette are used for an analysis. A blank containing 2 drops of leuco in 5 ml solvent should not produce an extinction exceeding 0.05 at 530 mμ (1 cm cuvette).

Determination of the extinction coefficient

In our first paper we reported that 0.001 milliequivalent of peroxide would give an extinction in 5 ml about 1.17. Hartmann and White [2], however, reported an extinction of 0.73, while Lea [3] recorded 0.83—0.84.

The extinction depends on the nature of the solvent. In the solvent mixture used in most of our experiments (benzene-ethanol-acetic acid, 45 : 50 : 5), careful determinations using several methods, as did Hartmann and White, gave an extinction of 1.00 at 520 mμ, and 1.02—1.03 at 530 mμ.

About the influence of the solvent the following can be said. If the extinction at the absorption maximum of a given concentration in ethanol is taken as unity, an extinction about 1.00 is also found with methanol, butanol, amyl alcohol, acetone, and ethyl acetate, but in solvents such as chloroform, benzene, and xylene, only about 0.60 is found (all solvents containing 2—5 % acetic acid). The presence of about 5 % ethanol in the nonpolar solvent will increase the extinction to about 0.8, and 15—30 % ethanol will bring it to about the same value as in ethanol. At the same time the addition of alcohol brings about a shift of the absorption maximum from about 500 mμ in xylene-acetic acid, to about 520 mμ, in the presence of 4 % ethanol, and to about 535 mμ at still higher alcohol concentrations. In our experiments we generally used absolute

ethanol for diluting our xylene-solutions to the mark in order to clarify them. This is the probable explanation of most of the discrepancy between our value and those found by the other workers, who use only 4 % butanol (0.2 ml color reagent) in their trials.

The presence of salts also may influence the extinction. When determinations are carried out in the presence of 10 % magnesium chloride, 0.001 milliequivalent peroxide will give an extinction of about 1.35.

The benzene-ethanol modification of the indophenol method

In our first paper we heated the xylene-acetic acid solution of leuco and fat in a boiling water bath for 10 minutes. Heating at 70° for 20 minutes is, however, sufficient to complete the oxidation of leuco by lipoperoxides. The greater part of the peroxide determinations in biological materials published by us were carried out using such a temperature and a solvent mixture consisting of ethanol-benzene-acetic acid 50 : 45 : 5.

The iron-catalyzed indophenol method

It is possible to carry out determinations at room temperature when a small amount of certain metals, for instance, iron or vanadium, is added. The determination can be carried out in the following way. The fat is dissolved in a mixture of equal parts of chloroform and alcohol to 5 ml volume. Two drops of the leuco reagent and one drop of a solution of 100 mg $FeCl_3$ in 100 ml of absolute alcohol are added, and the color compared with the blank after 10 minutes.

The indophenol method will give too high results when carried out in the presence of atmospheric oxygen. By the methods described above reproducible comparative results are obtained. In order to obtain correct values, however, it is necessary to carry out the determinations with the exclusion of oxygen. The iron-catalyzed indophenol method is especially easily carried out "anaerobically": The solution of fat + leuco is bubbled through with nitrogen for 10 minutes, a drop of ferric chloride is added, and the bubbling continued for 10 minutes more, after which the volume is adjusted and the colors are read in the photometer.

The presence of phospholipids causes an inhibition of the indophenol method. The inhibition can, in the case of the iron-catalyzed method, be overcome by the addition of magnesium chloride. Elevating its concentration results in acceleration of the color development. A method for the determination of peroxide groups in phospholipids by the indophenol method using the addition of 10 % magnesium chloride is reported in part C of this paper. The phospholipid inhibition of the iron-catalyzed indophenol method is probably due to complex-binding of iron to the phosphoric acid groups. A similar inhibition by phospholipids in the thiocyanate method for peroxide determination is probably also explained by complex-binding of the iron as will be reported in the following.

B. THE INFLUENCE OF PHOSPHATIDES ON THE THIOCYANATE METHOD

In a short communication Loftus Hills and Wilkinson [4] gave some observations on the influence of phosphatides on the determination of peroxide groups in fats by the ferric thiocyanate test. They added phosphatide in graded amounts to a series of solutions of 0.1 g of a slightly oxidized butterfat in 10 ml benzene-methanol. Milk phosphatide at a concentration of 0.05 % was found to interfere with the test, and in one of the experiments an amount of 0.5 % phosphatide completely inhibited color development.

These authors relate the effect of the phosphatides to the phosphoric acid group, pointing to the known interference by phosphoric acid in the thiocyanate test for ferric ions in aqueous solution. If this be true, *i.e.*, if complex formation between iron and the phosphoric acid groups of the phospholipids reduces the amount of red ferric thiocyanate compound, it is to be expected that the bleaching effect of phosphatide can be overcome by adding more thiocyanate or iron. This we found to be the case.

The first experiment, shown in Table 1, was carried out as described by Loftus Hills and Wilkinson. Graded amounts of phosphatide, freshly prepared from eggs, were added to a series of solutions of lard in 5 ml benzene-methanol (7 : 3). One drop of each of an ammonium thiocyanate and a ferric chloride solution, both prepared as described by Loftus Hills and Thiel [5], were added. The colors were read in the Beckman spectrophotometer at 500 mμ. As can be seen (Table 1), results almost identical with Loftus Hills and Wilkinson's with respect to the influence of phosphatide were observed. Next, 5 ml of a 6 % solution of ammonium thiocyanate in benzene-methanol was added to each test tube, and the colors read gain. The results as given in Table 1 are corrected for phosphatide blanks and adjusted to original volume of 5 ml. It is noted that even in the presence of 100 mg phosphatide, a large amount of ammonium thiocyanate could overcome most of the inhibition.

Table 2 shows the results of another experiment illustrating the effect of graded amounts of thiocyanate in overcoming the influence of a constant, high amount of phosphatide. A series of test-tubes were prepared containing

Table 1. The effect on the peroxide test of varying additions of phosphatide in the presence of small and large amounts of ammonium thiocyanate.

Weight of phosphatide added mg	Light absorption at 500 mμ	
	0.12 % NH_4SCN	3 % NH_4SCN
None	0.44	0.48
0.25	0.44	0.53
0.5	0.45	0.55
1	0.42	0.52
2.5	0.36	0.50
5	0.34	0.52
10	0.28	0.49
25	0.25	0.50
50	0.24	0.51
100	0.19	0.44

Table 2. The effect on the peroxide test of varying amounts of ammonium thiocyanate in the presence of a high amount of phosphatide.

Weight of ammonium thiocyanate added mg	Light absorption at 500 mμ
2.5	0.22
5	0.24
10	0.32
25	0.35
50	0.43
100	0.43
200	0.47
400	0.44

the same amount of a peroxide-containing lard as used in the previous experiment, 100 mg egg phosphatide, and the amount of ammonium thiocyanate given in the table, all in 5 ml benzene-methanol. One drop of the Loftus Hills-Thiel ferrous chloride solution was added, and the color read in the spectrophotometer.

Finally, an experiment is recorded in which the effect of increasing the iron concentration is demonstrated. The same solutions as in the first experiment — containing a constant amount of lard and graded amounts of phosphatide — were prepared in two series. One drop of the Loftus Hills ferrous chloride reagent per 10 ml solution was added to one series. One drop of a solution prepared in the same way and containing the same amount of hydrochloric acid, but twenty times as much iron, was added to the other series. The solutions were compared in the photometer with the corresponding blanks (Table 3).

The experiments recorded in Tables 1—3 quite well support the theory that the reason for the inhibiting effect of phosphatides in the thiocyanate test for peroxides is a complex-binding of ferric iron to phosphatides. This effect can be partly overcome by increasing the concentration of ferrous chloride added, or, more effectively, by increasing the thiocyanate concentration. In the Loftus Hills-Thiel method, the presence of phosphatide decreases the light absorption and also produces a shift in color from bright red to yellowish. This is possibly

Table 3. The effect on the peroxide test of low and high ferrous chloride concentration in the presence of varying amounts of phosphatides.

Weight of phosphatide added mg	Light absorption at 500 mμ	
	0.23 % $FeCl_2$ added	4.6 % $FeCl_2$ added
1.25	0.42	0.46
2.5	0.36	0.44
5	0.36	0.45
10	0.30	0.38
25	0.31	0.30
50	0.24	0.33
100	0.21	0.30

the reason that a still more complete inhibition is found when the colors are measured with a visual colorimeter of the type mostly used by Loftus Hills and his coworkers.

The experiments recorded in the tables were carried out in the same way as did Loftus Hills and Wilkinson, *i.e.*, without deaeration, but of course similar results are obtained when oxygen is excluded. The question whether the addition of high concentrations of ammonium thiocyanate can completely overcome the inhibiting effect of the phospholipids is discussed in the following part of this paper, which describes methods for the determination of peroxide groups in the presence of phospholipids.

C. THE DETERMINATION OF PEROXIDE GROUPS IN PHOSPHOLIPIDS AND PHOSPHOLIPID-CONTAINING FATS

Although it is well known that phospholipids usually contain a high amount of unsaturated fatty acids and are easily oxidized in the presence of atmospheric oxygen, it seems that the presence of peroxide groups in phospholipids has never been studied. Oxidation of the phospholipid has frequently been expressed simply by the decrease in iodine value.

Above we have reported our observations on the overcoming of the phospholipid inhibition in peroxide determination methods. The addition of magnesium chloride in the indophenol method, and of ammonium thiocyanate in the thiocyanate method, not only permit the determination of peroxide groups in triglycerides when phospholipids are present in large amounts, but also give reactions on oxidized samples of phospholipids containing very little triglyceride fat. The results obtained when the methods are employed on samples of phospholipids purified by repeated precipitation with acetone must be considered as indicative of the presence of peroxide groups in the phospholipids themselves.

Methods developed for the determination of peroxide in phospholipids and phospholipid-containing fats are outlined below.

Determination of peroxide groups in phospholipids by the indophenol method

A suitable amount of the lipid is dissolved in 5 ml of a mixture of equal parts of chloroform and 20 % $MgCl_2$, 6 H_2O in absolute ethanol. Two drops of leuco reagent and 1 drop of 0.1 % ferric chloride in ethanol are added. At the same time a blank containing 2 drops of leuco and 1 drop of ferric chloride solution in 5 ml chloroform-magnesium chloride-ethanol is prepared. After 10 minutes the colors are compared in the Beckman spectrophotometer at 530 mμ. The results are calculated on the basis of a linear relation between peroxide content and extinction of the amount of indophenol formed; 0.001 milliequivalent in 5 ml solution is assumed to give an extinction of about 1.35.

The test should preferably be carried out in the absence of oxygen. This can be done by passing oxygen-free nitrogen through the solution of lipid in magnesium chloride-containing solvent, and the corresponding blank for 10 minutes, then adding leuco and ferric chloride, and continuing to bubble with N_2 for 10 minutes. The deaeration can be carried out in the Beckman cuvettes using a volume of 3 ml and a glass capillary for the introduction of N_2.

Determination of peroxide groups in phospholipids by the thiocyanate method

A suitable amount of the lipid is dissolved in 5 ml of a solvent mixture containing 6 % ammonium thiocyanate in chloroform-ethanol (1 : 1). One drop of 0.22 % $FeCl_2$ prepared by Loftus Hills and Thiel's method is added. After a few minutes the solution is compared with a blank in the spectrophotometer at 500 mμ. The results are calculated on the supposition that 0.0003 milliequivalent peroxide in 5 ml solution gives an extinction about 1.0.

The test should be carried out in the absence of oxygen by passing purified N_2 through the solution for 10 minutes before ferrous chloride is added.

Results

A few typical results obtained by the two methods given above are presented in Table 4 which also contains the results found with the iodometric method. All methods were carried out in the absence of oxygen. The iodometric method used was the "cold" Lea modification [6], although this method has been worked out for triglycerides. The method is very unsuitable for phospholipids, since emulsions are formed when water is added before the titration, and at the same time a dark coloration of the oxidized phospholipids interferes with the exact observation of the disappearance of the iodine color. The results are, however, shown for comparison.

Table 4. Comparison of different methods for the determination of peroxide groups in phospholipids.

	Peroxide, milliequivalents/kg		
	Iodometric method	Indophenol method	Thiocyanate method
Egg phosphatides, freshly prepared	0	3.0	1.9
1 day old	0	5.1	5.0
3 days old	10	14.7	16.5
1 week »	12	20	23
1 month »	104	105	95
2 months old	250	320	340
Lecithin, *ex ovo*, Merck, about 10 years old	5	21	19

Discussion

From the reasons mentioned above, the iodometric method only permits very rough estimations. The results are probably too low, at least in freshly prepared phospholipids. On the other hand, with the two other methods, rather reproducible results are obtained that are almost identical, irrespective of the method.

In freshly prepared phospholipids generally higher values are found by the indophenol than by the thiocyanate method. The reason for this fact is not quite understood as yet. It might be that while in the indophenol method iron acts only as a catalyst, and so the final color development is independent of possible complex-binding of part of it to phosphoric acid groups, in the thio-

cyanate method partial binding of iron to phosphatides will lead to a decrease in color intensity. Such a difference between the functions of iron in the two methods could give a simple explanation of why too low values are found by the thiocyanate method in comparison with the, presumably, more stoichiometric values found by the indophenol method, when only small amounts of peroxide are present together with relatively much phospholipid. However, more work is needed to establish by which method the true peroxide content in the first stage of the oxidation of phospholipids is found. Until this problem is solved it cannot be definitely settled whether an upper limit exists for the amount of phosphatide that may safely be present in the thiocyanate method.

By means of the methods described we have been able to follow the formation of peroxides in freshly prepared phospholipids, and to demonstrate the presence of peroxides in certain phosphatides prepared from natural products. In a later paper methods for the separation of peroxides of phospholipids from peroxides of triglycerides will be described.

Since many fats and oils contain considerable amounts of phospholipids, Loftus Hills' modification of the thiocyanate method will yield too low values. When analyzing products like butter or soy-bean oil a higher concentration of thiocyanate should therefore be used, although perhaps not as high as in the case of products consisting almost exclusively of phospholipids.

Increasing the thiocyanate concentration up to a certain point gives a slightly higher light absorption of the ferric thiocyanate complex. A concentration of 2—5 % ammonium thiocyanate in the final solution will, in most cases, give maximal extinction.

As a solvent mixture we prefer a chloroform-ethanol solution. Although Loftus Hills and Thiel state that water has the same solubility in benzene-methanol as in chloroform-ethanol, we find that chloroform-ethanol (73 : 27) or chloroform-methanol will dissolve more water than the corresponding benzene-mixtures. Ethanol and, especially, methanol are good solvents for ammonium thiocyanate. Therefore, the use of aqueous solutions of this substance is not necessary.

As a result of these considerations we have arrived at the following modification of the thiocyanate method for fats containing moderate amounts of phospholipids. This modification can, of course, also be used for fats with low phospholipid content.

The fat is dissolved in chloroform-absolute ethanol (5 : 3) and brought to a volume of 8 ml. Two ml 30 % ammonium thiocyanate (analytical grade) in absolute methanol are added. After daeration for 10 minutes with purified nitrogen, 1 drop of the Loftus Hills-Thiel ferrous chloride reagent (or a reagent prepared in the same way but of double strength [7]) is added, and the colors are measured after a few minutes. In our hands, heating at 50° C was not necessary. Loftus Hills has come to the same conclusion (personal communication).

D. COMPARISON OF METHODS. GENERAL DISCUSSION

In this part of our paper we shall report typical results from experiments carried out in order to compare different methods for the determination of peroxides.

The iodometric method was carried out by the "cold" Lea method [6]. At the same time the substances were tested by the same method carried out "aerobically", that is, in exactly the same way but without bubbling through with nitrogen.

The thiocyanate method was used in the Loftus Hills and Thiel modification [5]. However, a double strength ferrous chloride solution was used [7], and the heating at 50° C was omitted. Also in this case two series of tests were carried out. In one series the solutions were deaerated with nitrogen for 15 minutes before the iron solution was added. In the case of benzoyl peroxide a higher concentration of ammonium thiocyanate (5 %) was used, since the concentration used by Hills and Thiel resulted in a very low reaction velocity.

Similarly, the same substances were tested with and without deaeration before the addition of ferric chloride, by the iron catalyzed indophenol method. The results are summarized in Table 5.

Table 5. Comparison of different methods for the determination of peroxide groups in fat, with and without the exclusion of atmospheric oxygen.

	Milliequivalents of peroxide per kg fat					
	Iodometric method		Thiocyanate method		Indophenol method	
	O_2 absent	O_2 present	O_2 absent	O_2 present	O_2 absent	O_2 present
Butterfat	0	0	0.2	0.25	0.3	0.46
»	0.6	1.2	1.25	1.45	1.1	1.75
»	1.07	2.2	1.85	2.1	1.75	2.45
Lard	4.3	4.7	5.8	7.6	4.0	7.4
»	280	344	395	450	320	570
Cod liver oil	5.4	6.4	6.6	7.1	4.8	9.6
» » »	28.5	36	42.5	49	39	52
Ethyl oleate hydroperoxide	840	940	970	1 230	840	1 140

It will be seen from Table 5 that all the methods give higher values in the presence of dissolved oxygen. In most cases the indophenol method showed the highest and the thiocyanate method the lowest degree of oxygen sensitivity.

The oxygen sensitivity of various methods has been studied by other investigators, *e.g.* Lea [3, 6, 9], who finds the iodometric method to be by far the least sensitive to oxygen. The reason for this discrepancy is, probably, smaller or larger differences in methods and purity of the chemicals. For example, Lea's studies on the influence of oxygen on the thiocyanate method were mainly carried out with a modification [9] rather different from ours.

It should be pointed out that we have found no considerable oxygen sensitivity in any of the methods when they were carried out on benzoyl or hydrogen peroxide. This also holds true for the original modification of the indophenol method first published [1]. Contrary to the criticism of Hartmann and White [2], our results suggest that atmospheric oxygen only interferes in the methods when a free radical is formed in the reaction which is stable enough to function as a carrier in a chain reaction.

When comparing the anaerobic results presented in Table 5, it is seen that, although in most cases the thiocyanate method gives the highest, the iodometric the lowest values, almost identical results are found, irrespective of the method.

The literature in this field is quite confusing, and direct comparisons between anaerobic methods for the determinations of peroxides in fats and oils are scarce. Lea [3], for instance, presents several tables comparing the aerobic colorimetric methods with the anaerobic iodometric method, but no figures concerning a direct comparison between the different anaerobic methods. He observed that ferric thiocyanate determinations with exclusion of atmospheric oxygen gave much too low results, but he presented no details that make it possible to find out why he was unable to obtain quantitative results with the thiocyanate method.

Kolthoff and Medalia [8] found lower results in certain oxidized fatty substances by the anaerobic thiocyanate method than by the iodometric method. However, they used a thiocyanate method very different from ours, with acetone as solvent.

Kolthoff and Medalia have proposed a mechanism for the thiocyanate reaction and possible by-reactions which explains the high results found by the influence of an organic solvent in the presence of oxygen, and low results due to decomposition by the solvent. They assume that the reaction between hydroperoxide and ferrous ions takes place through two one-electron transfers

$$ROOH + Fe^{++} \rightarrow RO^{\cdot} + OH^{-} + Fe^{+++};$$
$$RO^{\cdot} + Fe^{++} + H^{+} \rightarrow ROH + Fe^{+++}.$$

The free alkoxy radical RO $^{\cdot}$ is very reactive and may react with solvent molecules *etc.*, whereby the by-reactions are initiated.

The reactions are quite complex. Lea [3] can, however, scarcely be right in his rather pessimistic view that the complicated character *per se* of the reaction can prevent it from being made truly stoichiometric. The complicated character of the reaction so far cannot be used as an argument for the superiority of the iodometric method, especially since the chemical reactions involved in the iodometric method are probably equally complicated or quite analogous, with the intermediate formation of reactive alkoxy radicals.

Lea thinks that there is no advantage in carrying out the ferric thiocyanate determination with exclusion of atmospheric oxygen, since the method then loses much of its desirable simplicity, and also that the results with oxidized fats become much too low [3]. However, it seems that the thiocyanate method remains much simpler than the iodometric even though in both cases atmospheric oxygen is removed by bubbling with nitrogen. Lea [3] and G. Howard Smith [7] recommend the thiocyanate method in the presence of oxygen as a micromethod and think that by division with a conversion factor about 2, but slightly different for different fats, the values can be converted into the true values. Aerobic determinations carried out under uniform circumstance have proved to give relative values that are useful for many practical purposes. However, the physical foundation of the conversion factors involves so many single factors which can deviate from laboratory to laboratory and from trial to trial that their use becomes rather unsafe. Kolthoff and Medalia have

concluded, more justifiably, that the only sound basis for the development of satisfactory procedures for the thiocyanate reaction is complete exclusion of oxygen and the use of a solvent which entirely suppresses the induced decomposition of the peroxide.

When comparing the methods it should be borne in mind that the iodometric method is unsatisfactory for substances having a low peroxide content. The limit of sensitivity is about 0.05 ml 0.002 *N* thiosulfate, corresponding to 10^{-4} milliequivalent peroxide, probably a still higher value since a blank titration has to be subtracted. On the other hand, by the thiocyanate method, a difference in extinction of 0.05 can easily be observed, corresponding, in a 5 ml volume, to about 0.15×10^{-4} milliequivalent. For practical application, especially in work with foods, a high degree of sensitivity is the most important problem. In a product like butter, oxidative changes, giving a distinct off-flavor, are often observed by a peroxide content of about 0.2 milliequivalent per kg. Such a small quantity of peroxide can scarcely be determined by the iodometric method, whereas still lower values can quite well be determined by the colorimetric methods.

In nutritional work, *e.g.*, it is generally of little importance to know exactly the peroxide content of a product which has been definitely rancid for a long time, and there is thus little reason to use the rather troublesome iodometric method. Even though the colorimetric methods are only carried out with an error of about 5—10 %, this is, as a rule, sufficiently accurate for work with foods.

On the other hand, since a titration can be carried out very exactly, the iodometric method will allow much more exact determinations of higher peroxide contents. This is very important *e.g.*, in chemical work with peroxides, and the iodometric method must be preferred in such work.

A very sensitive method, based on the iodometric, is the thiofluorescein method, given by Dubouloz *et al.*[10]. However, while in the thiocyanate and the indophenol colorimetric methods, colors are measured that are proportional to the peroxide content, in the thiofluorescein method a decrease in color intensity is measured, so that the lowest contents of peroxide are least exactly determined. Furthermore, the thiofluorescein method is quite complicated in comparison with the other colorimetric methods.

Regarding the two colorimetric procedures, it must be concluded that the thiocyanate method is preferable to the indophenol method for general use. The reason for this conclusion is that although the very saturated indophenol color seems as intensive to the eye as the thiocyanate color, nevertheless a given amount of peroxide in the same volume will give a color intensity of only 30 % of the color intensity produced by the thiocyanate method with correspondingly lower sensitivity. For special purposes the indophenol method may still be useful, *e.g.*, in work with phospholipids, since in the first stage of the oxidation of such substances the indophenol method is possibly more truly stoichiometric.

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Effect of Irradiation on Hemin Formation

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Irradiation with Röntgen rays was found to depress very appreciably the rate of extrusion of ^{59}Fe bound to β_1-globulin in the circulation of the rabbit, the depression being much more pronounced 2 days after irradiation than shortly after exposure. It is suggested that erythrocytes of the bone marrow which have not yet completed their hemoglobin content are radioresistant and continue to incorporate ^{59}Fe in the bone marrow of the irradiated animal. Correspondingly, the effect of exposure to radiation on the rate of extrusion of ^{59}Fe from the plasma gets strongly pronounced only after the lapse of several hours.

A radiation dose which reduces the incorporation of ^{59}Fe into the hemoglobin to 30 % of that of controls does not diminish the rate of incorporation of ^{59}Fe into cytochrom b of the liver of guinea-pigs.

The rate of incorporation of ^{59}Fe into myoglobin of the muscles of guinea-pigs was found to be reduced in the exposed animals. The interpretation of this finding are discussed.

The study of iron metabolism can be approached in different ways, a very profitable one being the study of the fate of plasma iron. Iron utilized for hemoglobin formation has to pass the plasma, so iron coming from and going to the various organs in which it is present as a constituent of ferritin, hemosiderin, myoglobin, cytochrom, catalase or other compounds.

Flexner *et al.*[1] injecting labelled ferric chloride into the circulation of guinea-pigs observed a rapid initial disappearance of the radioactive iron from the circulation followed by a slower process. They interpreted the first process to be due to the disappearance of inorganic iron, the second to iron bound to proteins. These early experiments brought thus already out the difference between the circulating "physiological" iron and the plasma foreign iron injected into the circulation. The physiological plasma iron is bound to the β_1-globuline of the plasma which amounts to 3 % of the plasma proteins. This protein fraction, the transferrin, is identical with Cohn's fraction IV—7 as shown by Holmberg and Laurell [9], by Laurell [3] and others. That the albumine fraction of the plasma proteins does not fix iron was recently demonstrated by Wuhrmann and Jasinski [4] by combining paper chromatographic

with radioactive measurements. From the spots of the paper diagram of the plasma proteins from a rabbit 10 minutes previously injected with labelled ferric chloride only that due to β_1-globulin was found to be radioactive.

How the turnover rate of the circulating plasma iron is influenced by interference with hemeoipoetic processes going on in the human and animal organism was investigated in numerous cases by John Lawrence and his colleagues and several others.

Being interested in the problem of radiation anemia we determined the rate of disappearance of labelled plasma globulin from the circulation of rabbits exposed to Röntgen rays and the incorporation of ^{59}Fe into the circulating hemoglobin and into marrow hemin of guinea-pigs and rabbits. The effect of irradiation on the incorporation of ^{59}Fe into the red corpuscles of the rabbit and of the rat was previously investigated by Huff, Henessy and their associates [5,6].

In view of the marked effect of irradiation on the incorporation of ^{59}Fe into hemoglobin we wished to know if hemins formed in another *milieu* than the bone marrow are affected by irradiation as well. We investigated therefore the effect of exposure to radiation on the incorporation of ^{59}Fe into the iron containing enzymes of the liver and into cytochrome c and myoglobin of the muscles. In this note results obtained in the investigation of cytochrom b (Strittmatter [26]) of the liver and that of myoglobin are communicated.

EXPERIMENTAL

In 21 experiments with guinea-pigs an aggregate number of 220 animals, weighing 450—700 g, were injected intraperitoneally with 0.2—0.4 ml of saline containing 2 to 7 μg of with ^{59}Fe labelled iron of 1/2—1/4 μCurie activity. Half of the guinea-pigs was exposed to X-rays (160 kV, 43 r per min. for 10 to 33 minutes). Injection took place 15 to 300 min. following exposure. No food was administered during the experiment.

The animals were killed 4 to 48 hrs later, their livers homogenized in sucrose and fractionated as described by Hogbein and Schneider. Ferritin was extracted both from microsomes and from the supernatant, cytochrom b hemin from microsomes (Loftfield [7]) and catalase from the supernatant. Total iron, cytochrom c and myoglobin iron were furthermore isolated from the muscles making use of the method of Theorell and Åkeson [8].

Ferritin was precipitated from the supernatant by addition of half volume of ammoniumsulfate. The precipitate was taken up in water and repeatedly fractionated with ammoniumsulfate. The precipitate was then taken up in water, heated to 70° C for a few minutes and centrifuged after cooling. The supernatant ferritin was found to contain 17 to 20 % iron.

The microsomes were homogenized in water, the pH adjusted to 5 and centrifuged after the lapse of 30 minutes. The precipitate was once more homogenized and washed with water. All the microsome ferritin was found to be in the water phase. The insoluble particles contained the cytochrom b and some proteinbound iron. The cytochrom b hemin was extracted from this fraction with a mixture of acetone and HCl (10 ml 20 % HCl in 1 liter of acetone). The acetone was evaporated *in vacuo*, the precipitated hemin washed with 1 *N* HCl and dissolved in ether. The ether was evaporated and the hemin combusted with sulfuric acid and hydrogen peroxide. The determination of iron was carried out by a colorimetric method using the sulfosalicylic acid complex.

An aliquot of the solution was used for colorimetric determination of the iron content, to another aliquot iron chloride was added bringing its iron content up to 500 μg. The iron was precipitated as FeS and filtered through a perforated aluminium dish covered with filter paper as described by Agner *et al.*[9]. The aluminium dish was then placed under the window of a Geiger counter. An aliquot of the solution which was injected was trea-

ted in the same way. The data obtained permitted to calculate the relative specific activity of the iron fraction (activity per mg iron in arbitrary units) and also the absolute specific activity (percentage of injected ^{59}Fe present in 1 mg of iron).

In our experiments on the iron turnover of the plasma 5—10 μg of iron having an activity of about 2 μCurie were injected into the ear vein of a rabbit. After the lapse of about 1 h a few ml of the plasma of this rabbit, which now contained all ^{59}Fe present in the plasma as β_1-globulin, was injected into the jugular vein of a sister rabbit. At intervals blood samples were taken from the carotic vein of this rabbit, the heparinized blood centrifuged, the plasma combusted as described above, 500 μg carrier iron added and the iron precipitated as sulphide as described above. 2—3 kg rabbits were applied in these experiments, in which the effect of irradiation on the formation of labelled hemoglobin and labelled hemin of the bonemarrow was investigated.

RESULTS

a) Cytochrom b

The data obtained for the ratio of the specific activities of cytochrom b iron (Strittmatter [26]) and also of microsome ferritin iron of irradiated and control guinea-pigs are listed in Table 1. Out of 13 ratios listed in Table 1 one is as low as 0.77, but even this ratio is by about 250 % higher than that obtained for hemoglobin iron, which was found to be 0.3, corresponding to a depression of ^{59}Fe incorporation into hemoglobin in the irradiated guinea-pig to 1/3 of that into the hemoglobin of controls.

Table 1. Effect of exposure to 500—1 400 r on the incorporation of ^{59}Fe into the cytochrom b of the liver of guinea-pigs. Each figure is obtained by pooling 5 livers.

Time between injection of ^{59}Fe and removal of the liver in hrs	Ratio of spec. activ. of cytochrome b iron of irradiated and control animals	Ratio of spec. activity of microsome ferritin iron of irradiated and control animals
4	2.52	1.62
4	0.77	1.12
4	0.96	1.05
16	1.17	0.91
17	1.48	1.35
17	0.88	1.16
17	0.92	1.06
17	1.90	1.39
17	1.15	1.40
48	1.12	0.94
48	1.16	1.19
48	1.25	1.81
48	0.94	2.13
Mean value	1.25 ± 0.036	1.32 ± 0.010

Not only does exposure of guinea-pigs to large doses of Röntgen rays not diminish the rate of incorporation of the ^{59}Fe into cytochrome b but as seen in Table 1 promotes it. The increased incorporation of ^{59}Fe does, however, not or not mainly indicate an increased rate of formation of cytochrome b. It is, at least to a large extent, due to the change in sensitivity of the radio-

active indicator. About half of the plasma iron takes its way into the bone marrow and is applied to hemoglobin synthesis, the other half into the liver and other organs. The labelled iron atoms which leave the plasma are replaced by inactive ones coming from the organ depots and to a minor extent from the intestine. Irradiation upsets this distribution. Incorporation into hemoglobin is strongly reduced which results in an increased life time of ^{59}Fe in the plasma which in turn leads to a change in the sensitivity of the radioactive indicator. The increase in the iron content of the plasma of the irradiated animals (Schuck [10], Ludewig [11]) remains behind the increase of its ^{59}Fe content and correspondingly the sensitivity of the radioactive indicator decreases. Thus we can expect more ^{59}Fe to reach the liver in the exposed animals than in the controls. If this line of thought is correct we must expect the ratio of the specific activity of the iron of exposed and control animals, which for cytochrom b was found to be 1.3, to be about 1.3 for other liver fractions as well (as far as their rate of formation is not influenced by irradiation). As seen from Table 1 this is almost the case for microsome ferritin, 1.32 being found for the corresponding ratio.

Elmlinger *et al.*[12] found in normal humans about 55 % of the iron which passed the plasma to be utilized in red corpuscle formation. The rest takes its way into the other organs. Should the last mentioned inflow be unilateral the iron content of these organs would increase already in the course of 120 days with about 2 g which is more than the total iron depot of a man which amounts to about 1.6 g (Haskins [13]). We have therefore to conclude that the flow or iron from the plasma into the depot organs goes hand in hand with an outflow of an even larger amount of iron from the depots into the plasma.

A different line of thought supports this view. Hemoglobin iron is almost quantitatively reutilized for hemoglobin formation as shown by Hahn *et al.*[29]. We injected labelled red corpuscles of a donor rabbit to an acceptor rabbit and after the short interval of only 5 hours found 90 times more ^{59}Fe in the liver than in the spleen of the thoroughly perfused animals, the gall containing 2/3 of that of the last mentioned organ. The reutilization of this iron for hemoglobin formation necessitates its passage through the plasma to the marrow. Thus a continuous flow of iron from the liver (and presumably other organs responsible for the phagocytosis of red corpuscles) to plasma has to take place. In the course of 120 days (the time necessary to replace the circulating human red corpuscles) about 2 g of iron has to pass from the depot organs to the bone marrow. The corresponding figure in the rabbit in the course of 50 days is about 0.1.

Effect of irradiation on ^{59}Fe incorporation into hemoglobin. As to be expected and also shown by Huff [5], Hennessy [6] and their associates irradiation strongly depresses the incorporation of ^{59}Fe into hemoglobin. Our results obtained with guinea-pigs injected within 6 hours after exposure and killed 17 hours later are seen in Table 2.

In each of the experiments Nos. 9 to 16 the blood of 10 guinea-pigs, in Nos. 21 and 22 of 20 animals was pooled.

Incorporation of ^{59}Fe into hemoglobin was thus reduced by irradiation in these experiments to 30 % of that of controls.

Table 2. Effect of irradiation with 500—1 300 r on the incorporation of ^{59}Fe into hemoglobin of guinea-pigs.

Number of experiment	Ratio of spec. activ. of hemoglobin iron of irradiated and control guinea-pigs
9	
10	0.20
12	0.17
13	0.36
14	0.72
15	0.25
16	0.21
21	0.29
22	0.24
	0.28

Mean value: 0.298 ± 0.158

Delay of the effect of irradiation on the incorporation of labelled iron into hemoglobin. Red corpuscles are very radioresistant. We need very massive doses to achieve hemolysis and a dose of 4 000 r for example as shown by Sheppard [14] does not influence the rate of intrusion of ^{42}K in the red corpuscle though it does to some extent weaken the mechanism responsible for the concentration of potassium in the red corpuscles. Dog blood exposed to a dose of 200 000 r does not hemolyse as observed by Nizet *et al.*[24].

The radioresistance of circulating red corpuscles suggests that the not fully completed red corpuscles in the bone marrow are radiation resistant as well and continue even in the exposed animal to complete their hemoglobin content. In the bone marrow of swine exposed to radiation of the Bikini explosion often only fat cells and a few clumps of erythrocytes were found [25].

The above conclusion is supported by the observation that the uptake of ^{59}Fe or ^{14}C by the hemoglobin of red corpuscles *in vitro* is not reduced by irradiating the animal before securing the blood sample. Reticulocytes and other types of incomplete red corpuscles can complete their hemoglobin content *in vitro*. Not only is the ^{14}C incorporation *in vitro* not smaller into the blood corpuscles of irradiated animals than into those of controls, it is even markedly enhanced as shown by Nizet *et al.*[15] who investigated the uptake of ^{14}C by the dog. Rauntanan and one of us made similar observations when studying incorporation of ^{59}Fe *in vitro* into the red corpuscles of the hen which was found to be increased in extreme cases up to 300 % if the hen was 18 hours previously exposed to a dose of 1 000 r. Irradiation induces presumably the bone marrow to release some of the red corpuscles in an earlier stage of their maturation. Nizet *et al.* have furthermore shown that irradiation *in vitro* produces changes in the plasma which are favourable to ^{59}Fe incorporation into the red corpuscles.

These considerations support the conclusion that as far as red corpuscles in the advanced stage of their maturation are present in the bone marrow a completion of their hemoglobin content takes place even in the exposed organism

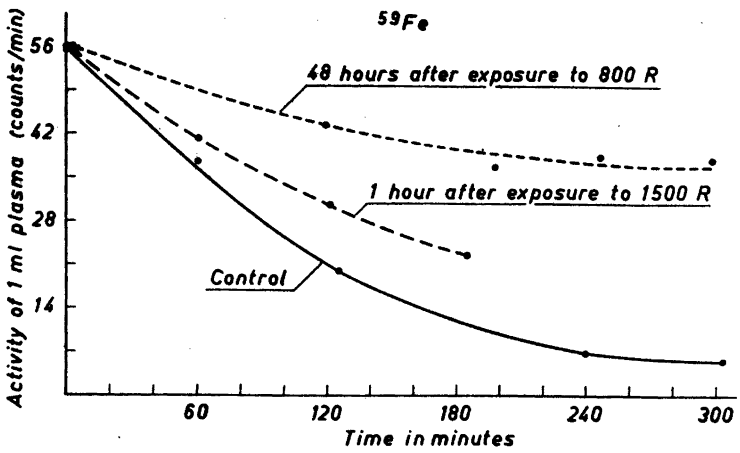

Fig. 1. Effects of exposure to Röntgen rays on the rate of extrusion of circulating labelled plasma iron from the circulation of rabbits.

and that some of the ^{59}Fe administered shortly after irradiation will be utilized by the bone marrow. With increasing time the completed red corpuscles being discharged into the circulation or wiped out in the exposed organism the utilization of ^{59}Fe by the bone marrow will diminish and after the lapse of about 1 day may almost cease. As the non-utilization of labelled plasma iron by the bone-marrow leads to a depressed turnover rate of the plasma iron this depression is to be expected not to be shown to its full extent already shortly after irradiation but only at a later date. That this is the case is demonstrated by Fig. 1 and the data of Table 2.

As seen in Fig. 1 the effect of irradiation with such a high dose as 1 700 r influences the rate of extrusion of ^{59}Fe from the plasma, when measured 1/2 hour only after exposure, is much less than an irradiation with 800 r only, when the rate of extrusion is measured 2 days after irradiation.

When injecting rabbits weighing 600 g intraperitoneally with labelled $FeCl_3$ 48 hours after exposure to 500 r we observed 5 hours after injection the ^{59}Fe content of the plasma to be 120 % higher than that of the control. In experiments in which 500—900 g rabbits were injected intraperitoneally within 1/2 hour after irradiation with 5 μg labelled iron as $FeCl_3$ and killed 3 hours later the ^{59}Fe content of the plasma was increased with 6 % only. Further data on the effect of time which elapsed after exposure to irradiation on the ^{59}Fe content of the plasma is seen from Table 3. This table also contains data on the incorporation of ^{59}Fe into hemoglobin, which is much more markedly depressed under the effect of irradiation after the lapse of 48 hours than after that of 1/2 hour. In these experiments 5 μg of iron as $FeCl_3$ were interperitoneally injected to each rabbit. Similar observations on the effect of irradiation on the incorporation of ^{59}Fe into the red corpuscles were made by Hennessy and Huff[6].

These results support the conclusion arrived at that for some time after irradiation the completion of the hemoglobin content of the red corpuscles is still going on in the marrow and supports indirectly also the result arrived at by Thorell that hemoglobin is layed down in the later phase of the maturation of erythropoeitic cells of the bone marrow.

Table 3. Incorporation of interperitoneally injected ^{59}Fe in the course of 5 hours into the hemoglobin of controls and with 500 r irradiated rabbits. C = control. R = exposed.

Time in hours between exposure and injecting of ^{59}Fe	Relative ^{59}Fe content			
	Plasma		Hemoglobin	
	C	R	C	R
0	100	100	100	100
0.5	100	118	100	68 *
17	100	124	100	56
48	100	223	100	3.2
72 **	100	447	100	17.3

* For the bone marrow hemin the corresponding figure was 43.
** Data obtained by Huff *et al.* [10]

b) Muscle myoglobin

In view of the fact that the chemical composition of myoglobin closely resembles that of hemoglobin — they are differing only as to their degree of polymerisation — it was of great interest to investigate the effect of exposure to irradiation on the incorporation of ^{59}Fe into the myoglobin of guinea-pigs extracted from their muscles. The method of purification was that described by Theorell and Åkeson [8]. The myoglobin obtained was practically free from hemoglobin as revealed by a spectroscopic investigation of the CO-myoglobin. In 4 experiments in each of which 10 female guinea-pigs having a weight of 450—550 g were injected 6 hours after exposure to 1 400 r with 3 μg of labelled iron as sodium iron citrate and killed 18 hours later and 10 controls were treated in the same way the following rel. specific activity figures were obtained for the myoglobin and hemoglobin iron of controls and exposed animals.

Table 4. Effect of irradiation on the incorporation of ^{59}Fe into myoglobin and hemoglobin of guinea-pigs. Control = C, exposed = R.

No. of expt.	Rel. spec. activity * of myoglobin		Ratio $\frac{C}{R}$	Rel. spec. activity of hemoglobin		Ratio $\frac{C}{R}$
	C	R		C	R	
1	0.098	0.042	2.3	0.236	0.047	5.0
2	0.034	0.026	1.3	—	—	—
3	0.476	0.215	2.2	2.19	0.48	4.6
4	0.415	0.164	2.6	2.44	0.68	3.6

* count per μg Fe.

Under effect of exposure the ^{59}Fe incorporation into myoglobin was thus reduced to 1/2, that into hemoglobin to 1/4 of that of the controls. In the 2 last experiments we determined the effect of exposure on the specific activity of the total iron present in the muscles as well and found it, as to be expected (*cf.* p. 511), increased under the effect of exposure, in the third experiment from 0.714 to 0.810, in the fourth one from 0.687 to 1.04.

DISCUSSION

Hemoglobin is one of the comparatively few molecular constituents of the adult organism that is formed in close connection with cell division. The latter being very susceptible to the effect of ionising radiation, exposure to radiation is bound to interfere with hemoglobin formation as well. Furthermore marrow cells are radiosensitive and exposure to radiation may lead to destruction of marrow cells. Irradiation of rats with 400 r was found to lead to a decrease in the total number of marrow cells to almost one half of its normal value in the course of the first day following exposure (Brecher [15]) and the chemical composition of the bone marrow was found markedly influenced. Lutwak-Mann [17] found 3 hours only after total body exposure to a dose of 1 500 r the labile acid-soluble P of the bone marrow of rabbits to be reduced by 30 %, that of DNA and RNA phosphorus by 20 and 16 %. The total nucleic acid P of the marrow of the to 500 r exposed rat amounted to only half of that of controls. Mandel *et al.*[18] report the bone-marrow of rats exposed to 500 r to show after the lapse of 26 hours a by 50 % reduced PNA content, the DNA being reduced to 30 % of that of the controls after the lapse of 2 days. Thus radiation anemia is at least partly due to the fact that hemoglobin is formed in close connection with the cell division and also that it takes place in the radiosensitive marrow cells.

Altmann *et al.* (Richmond [19], Stokinger [20]) in their very extended studies found incorporation of ^{14}C into globin and hemin to be influenced at a very different rate by irradiation. Incorporation of ^{14}C into hemin of the exposed animals was greatly depressed, whereas globin was affected to a considerably smaller extent. From this finding it does not necessarily follow that it is not the *milieu* of its formation, the bone marrow, which is responsible for the radiation sensitivity of the formation of hemoglobin.

We found no interference with the formation of cytochrom b in the liver of the strongly irradiated guinea-pig. In this case heme formation is thus not radiosensitive. We found, however, interference with formation of myoglobin. We are reluctant to make any definite conclusion from this finding. The possibility must not be excluded that myoglobin formation in the muscles of the 450—550 g guinea-pig takes place in connection with mitotic processes. Histologic evidence proves cell division in muscle tissue to be a very rare process. A very few mitoses would however be sufficient to account for the formation of much of the myoglobin of the muscles as a process preceded by cell division. This is shown by the following calculation.

The maintenance of a hemoglobin level of 6 g per kg guinea-pig requires a mitotic figure of 1—2 % in the hemopoietic bone marrow weighing about 10 g. The amount of myoglobin present in the guinea pig is not known. Assuming

the hemoglobin content of the guinea-pig to be 32 times its myoglobin content as found for the rat by Drabkin [27] and furthermore that the muscle tissue of the guinea-pig amounts to 40 % of its body weight, *i.e.* its myoglobin being formed in a 40 times larger amount of tissue than its hemoglobin, then the mitotic figure to maintain the production of myoglobin should be 32 × 40 times less than that permitting the production of hemoglobin. We have further to consider that the life cycle of myoglobin is longer than that of hemoglobin. No exact data are available on the life cycle of myoglobin. Theorell *et al.*[28] estimate hemoglobin to have an about 4 times longer life time than hemoglobin and from the finding of Helwig and Greenberg [21] a ratio of 2—3 follows for the life time of the protohemin part of myoglobin and hemoglobin; our figures suggest a ratio of about 3. We have thus to multiply the above mentioned 32 × 40 by at least 3 to arrive at the ratio of mitotic figures in the hemopietic bone marrow and the muscle tissue assuming the formation of myoglobin being preceded by cell division. This ratio works out to be about 4 000. If out of 200 000 cells one would show division it would suffice to interpret myoglobin formation to be in close connection with cell division.

It is often assumed that amiotic division of muscle cells is less rare than a miotic one. As irradiation can be expected to interfere with this type of cell division as well it is bound to depress myoglobin formation if myoglobin is laid down as we are inclined to assume in the course of maturation of muscle cells. We are engaged in determining the effect of irradiation on the incorporation of ^{59}Fe into myoglobin of both fully grown and growing rats in the hope of getting more information on the radiation sensitivity of myoglobin formation.

Our thanks are due for valuable assistance to Mr. Åke Åkeson, Mrs. Ingegerd Björkman and Miss Jutta Schliack, to the *Knut och Alice Wallenbergs Stiftelse* and to the *Riksföreningen för kräftsjukdomarnas bekämpande, Cancernämnden*, for their generous support of this investigation.

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Synthesis of Ureidosuccinic Acid with Soluble Enzymes from Liver Mitochondria and *Escherichia coli* *

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An extract from rat liver mitochondria catalyzed the enzymatic formation of ureidosuccinic acid from aspartic acid, NH_3 and CO_2 in the presence of acetylglutamate, Mg^{++}, ATP and an ATP regenerating system. It was shown that the reaction proceeded in two enzymatic steps. The first involved the formation of compound X from acetylglutamate, NH_3, CO_2 and ATP in the presence of Mg^{++}. In the second step compound X was condensed with aspartate to form ureidosuccinate.

The enzyme catalyzing the second step was also present in rat spleen, kidney and heart and in an extract of *E. coli*.

The enzyme from *E. coli* was purified about tenfold and found to catalyze the following reaction:

L-aspartate + compound X → L-ureidosuccinate (+ acetylglutamate + phosphate).

The enzymatic synthesis of ureidosuccinic acid (USA **) has been demonstrated with isolated mitochondria from rat liver. [1] It was found that USA was synthesized from NH_3, CO_2 and L-aspartate in the presence of Mg^{++}, ATP and carbamyglutamate under conditions where ATP was regenerated by oxidation of glutamate or succinate.

The requirement of a substituted glutamate derivative suggested a similarity between USA synthesis and the enzymatic synthesis of citrulline, which is also localized in rat liver mitochondria. The work of Grisolia and Cohen (see *e.g.* Grisolia [3]) has demonstrated that the enzymatic synthesis of citrulline requires at least two steps. The first involves an "activation" of CO_2 and NH_3 with a substituted glutamate derivative (*e.g.* carbamylglutamate or acetylglutamate) and ATP in the presence of Mg^{++} to form an unstable "compound X" whose exact structure has not yet been elucidated:

* A preliminary report of part of this work was given at a meeting of the Swedish Biochemical Society (*Acta Chem. Scand.* 8 (1954) 1102).

** The following abbrevations are used in this paper: USA, ureidosuccinic acid; ATP, adenosine triphosphate; PGA, 3-phosphoglyceric acid; PCA, perchloric acid; MP, muscle protein fraction, prepared according to Ratner and Pappas [2].

$$NH_3 + CO_2 + ATP + \text{acetylglutamate} \rightarrow \text{compound X} + ADP \quad (1)$$

In the second reaction citrulline is formed by condensation of compound X with ornithine:

$$\text{Compound X} + \text{ornithine} \rightarrow \text{citrulline} + \text{acetylglutamate} + P_i \quad (2)$$

USA synthesis also involves a fixation of ammonia and carbon dioxide on an amino group which is carried out with the aid of carbamyl- or acetylglutamate and ATP [1]. It seemed rather probable, therefore, that reaction (1) was the first step in USA synthesis also, followed in sequence by the final reaction:

$$\text{Compound X} + \text{L-aspartate} \rightarrow \text{L-ureidosuccinate} + \text{acetylglutamate} + P_i \quad (3)$$

The present paper describes evidence obtained for reactions (1) + (3) in extracts from rat liver mitochondria. Reaction (3) has furthermore been studied with a partially purified enzyme from *E. coli*.

After the completion of this work Lowenstein and Cohen [4] have briefly described the formation of labeled USA from ^{14}C-compound X in rat liver supernatant.

MATERIALS AND METHODS

L-Acetylglutamic acid was synthesized according to Karrer *et al.*[5], *D,L-ureidosuccinic acid* according to Nyc and Mitchell [6]. *ATP* and *PGA* were purchased as barium salts from Sigma company. The sodium salts were prepared as described earlier for ATP [1].

L-Aspartic acid-^{15}N and *L-aspartic acid-2,3-*^{14}C have been synthesized earlier [7].

Acetone powder of rat liver residue and *enzyme B* (obtained by alcohol fractionation of the acetone powder extract) were prepared as described by Grisolia and Cohen [8].

Compound X was prepared enzymatically and purified according to an unpublished method of Grisolia *. It was found that optimal time conditions for obtaining compound X synthesis had to be determined for each preparation of enzyme B. As already demonstrated by Grisolia and Cohen [8] the amount of compound X formed reached a maximum after some time and then rapidly decreased. Increasing enzyme concentration produced compound X in maximal yields of 40—50 % (based on acetylglutamate). Further increases in enzyme concentration served only to produce a more rapid formation of about the same amount of compound X. These interrelationships are demonstrated by Fig. 1.

For each new enzyme preparation a time curve of optimal compound X synthesis was determined with an enzyme concentration of 10 mg/ml. The same enzyme preparation gave then very reproducible results.

Purification of compound X was carried to the "20 % pure" stage according to Grisolia's description with isolation as the calcium salt. According to the enzymatic assay (formation of citrulline with heat denatured enzyme B and ornithine [8]) different preparations contained between 16.5—21 % compound X. A nitrogen analysis in one case showed that 62 % of the nitrogen in the calcium precipitate corresponded to compound X. When $^{15}NH_4Cl$ was used for the preparation of compound X, isotope analysis of the "20 % pure" precipitate showed that 42 % of all nitrogen originated from the isotopic ammonia. Pure compound X would give a value of 50 %, since only one out of two nitrogen atoms stems from ammonia. The data thus indicate that a greater part of the impurities in the "20 % pure" precipitate do not contain nitrogen. Light absorption measurements of an extract of compound X showed only negligable absorption at 2 600 Å (corresponding to less than one molecule of adenine derivative per 100 molecules of compound X).

* We wish to express our great appreciation to Dr. Grisolia who gave us the details of the method for preparation of compound X before publication.

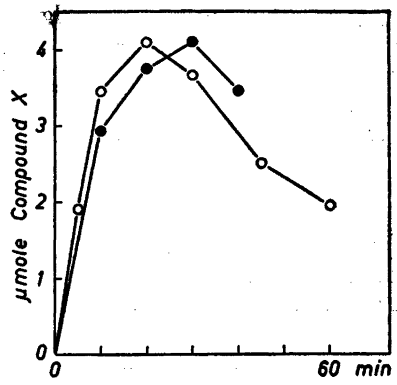


Fig. 1. Synthesis of compound X. Each vessel contained per ml: 2 µmole ATP, 25 µmole PGA, 20 µmole $MgSO_4$, 10 µmole acetylglutamate, 15 µmole $NaHCO_3$, 20 µmole NH_4Cl, 2 mg MP. pH = 7.3. ○ = 15 mg enzyme B, ● = 10 mg enzyme B. The values for the formation of compound X are given for 1 ml.

Quantitative analysis for compound X was carried out with heat treated enzyme B[8]. Analysis for citrulline was made according to Koritz and Cohen [9].

Determination of ureidosuccinate. The experiments were carried out with isotopic substrates. In the study of the liver enzyme $^{15}NH_4Cl$ and L-aspartate-^{15}N were used. The amount of USA formed was then determined by the isotope dilution method as described earlier [1]. In experiments with the enzyme from *E. coli* L-aspartate-2,3-^{14}C was the substrate together with nonlabeled ammonia and CO_2, or compound X. At the end of each experiment carrier nonlabeled USA (usually 200 µmoles) was added and the amount of enzymatically formed USA was calculated from the radioactivity in the reisolated substance. Radioactivity determinations were carried out on infinitely thin samples in a windowless gas flow counter. Since the aspartic acid used as substrate contained 12 000 ct/min./µmole (infinite thin sample) the amount of enzymatically formed USA could be calculated according to:

$$\mu\text{moles USA formed} = 200 \cdot a/12\,000 = a/60$$

where a = ct/min/µmole in the reisolated USA.

RESULTS

USA synthesis in mitochondrial extracts

Preparation of enzyme. Rat or rabbit liver mitochondria were prepared as described earlier [1] by differential centrifugation according to the principles worked out by Schneider and Hogeboom [10]. The enzymes synthesizing USA could be obtained in solution after rupture of the mitochondrial membrane. This could be done by suspension of the washed mitochondria in distilled water or by sonic vibration. The latter treatment, however, rapidly inactivated the enzymes, while following osmotic rupture more than 50 % of the enzymatic activity remained in the sediment after centrifugation of the lysed mitochondria. The best yield of soluble enzymes was obtained by a quick freezing and thawing of a mitochondrial suspension in 0.1 *M* $NaHCO_3$ (10—20 ml solution per 10 g of liver). When the resulting suspension of broken mitochondria was centrifuged at 20 000—100 000 g for 20 minutes at 0° most of the activity was located in the supernatant. Not quite as good results were obtained using 0.1 *M* potassium phosphate buffer, pH 7.5, or isotonic KCl as the suspending solution. Freezing in distilled water destroyed most of the activity. Repeated freezing and thawing in bicarbonate also decreased the activity.

Routinely the enzyme was prepared in 0.1 *M* $NaHCO_3$. The clear supernatant was filtered through cotton wool to remove fat particles and could then be lyophilized and stored as a dry powder in a dessicator at —10°. Enzymatic activity invariably decreased during storage, although there was considerable variation in the rate of decline of activity with different preparations. More than 50 % of the initial activity was usually retained for 2 weeks.

Most of the experiments were carried out with rat liver mitochondrial extracts, which contained the highest enzymatic acitivities (per amount of nitrogen). Some experiments with rabbit liver mitochondrial preparations were included, although here more variation in activity among preparations was encountered.

Table 1. Requirements for USA synthesis in mitochondrial extract.

	μmole ureidosuccinate formed
complete substrate	0.70
— aspartate	0.01
— ammonia	0.07
— $MgCl_2$	0.02
— ATP, PGA, MP	0.00
— PGA, MP	0.15
— acetylglutamate	0.04

Complete substrate: 30 μmole L-aspartate-^{15}N, 30 μmole $^{15}NH_4Cl$, 25 μmole $MgCl_2$, 10 μmole ATP, 50 μmole PGA, 15 μmole acetylglutamate, 100 μmole phosphate buffer pH 7.2, 3 mg MP. Volume = 3 ml. All acids neutralized with KOH. Incubation for 90 minutes at 37° with 1 mg enzyme nitrogen.

Overall requirements for USA synthesis. USA was synthesized from the same substrates by the extracts as by the intact mitochondria and also required the presence of acetylglutamate (or carbamylglutamate), Mg^{++} and ATP (Table 1). Acetylglutamate was used throughout the present investigation rather than carbamylglutamate, which had been used with intact mitochondria [1], since it was found that smaller concentrations of acetylglutamate gave optimal USA synthesis with intact mitochondria. This is in accordance with the results obtained by Grisolia and Cohen [11] on citrulline synthesis.

In the absence of acetylglutamate USA synthesis was reduced from 0.70 μmole to 0.04 μmole under the experimental conditions of Table 1. The catalytic nature of acetylglutamate requirement in the overall reaction could be demonstrated in an experiment, in which only 0.5 μmole of acetylglutamate was present in the substrate. During 6 hours incubation 2.1 μmole of USA was formed. Except for acetylglutamate conditions were as in Table 1. Another 50 μmole of PGA was added after 3 hours.

In the absence of ATP no USA synthesis was obtained with rat liver mitochondrial extracts and an otherwise complete substrate (Table 1). ATP was broken down during the reaction by ATP-ases in the extract. For optimal synthesis of USA, 3-phospho-glyceric acid and a muscle protein fraction had to

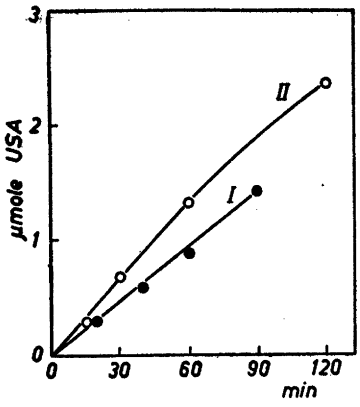


Fig. 2. Time curve for USA formation in mitochondrial extract. Curve I = overall reaction, curve II = formation from purified compound X.
*Substrates: Curve I: 60 µmole L-aspartate-^{15}N, 60 µmole $^{15}NH_4Cl$, 40 µmole acetylglutamate, 40 µmole ATP, 200 µmole PGA, 12 mg MP, 100 µmole $MgCl_2$, 120 µmole phosphate buffer, pH 7.2. Volume = 12 ml. 4.1 mg enzyme nitrogen. Curve II: 25 µmole L-aspartate-^{14}C, 22 µmole compound X *, 500 µmole phosphate buffer, pH = 7.0. Volume = 15 ml. 4.8 mg enzyme nitrogen.*

be added in order to regenerate ATP. The same general type of behaviour has been found first by Ratner in arginine synthesis [2] and also by Grisolia and Cohen [8] in citrulline synthesis. In both cases the accumulation of ADP formed by ATP breakdown inhibited the reaction. Ratner showed that this difficulty could be overcome by the addition of PGA which in the presence of a suitable ammonium sulfate fraction from a muscle extract regenerates ATP from ADP.

The *time curve* of the overall reaction is given by Fig. 2, curve I. The influence of *enzyme concentration* is demonstrated by Fig. 3.

Stepwise synthesis of USA. An extract from acetone powder of liver residue [8] which showed good activity for citrulline synthesis formed very little USA under the present conditions. The mitochondrial extract, on the other hand, could synthesize either citrulline or USA. This marked difference between the two preparations in the relative ability to synthesize citrulline and USA (summarized in Table 2) demonstrates that at least one enzyme in USA synthesis is different from the enzymes in citrulline synthesis.

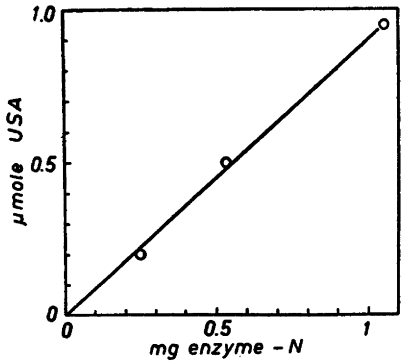


Fig. 3. Overall formation of ureidosuccinate with different amounts of enzyme. Substrate as in Table 1. Incubation for 90 min. at 37°.

* *The finely divided Ca-salt of compound X was extracted for two minutes with ice cold water (2—8 ml of water/50 mg Ca-salt), a slight excess of potassium oxalate (M solution) was added and the suspension centrifuged in the cold. Aliquots of the solution were immediately used for a serie of experiments*

Table 2. Non identity of enzymes for citrulline and ureidosuccinate synthesis.

	Synthesized per mg enzyme nitrogen	
	μmole of citrulline	μmole of ureidosuccinate
Acetone powder extract:		
30 min. incubation	0.97	0.05
90 min. incubation	1.92	0.04
Mitochondrial extract:		
30 min. incubation	1.03	0.40
90 min. incubation	1.53	0.89

Substrates as in Table 1 for ureidosuccinate synthesis. In the citrulline experiments 20 μmole of $NaHCO_3$ was added to each medium and 20 μmole ornithine was substituted for L-aspartate.

Table 3 demonstrates that more than one enzyme is involved in USA synthesis. When the mitochondrial enzyme was heated at 54° for 3 minutes (pH 7.4) its ability to synthesize USA was almost completely destroyed. This treatment has been shown to inactivate the first step in citrulline synthesis [8]. The combination of this heated mitochondrial enzyme and acetone powder extract, each of which was virtually inactive alone, synthesized USA actively (Table 3). The addition of heated mitochondrial enzyme to a non heat-treated mitochondrial enzyme increased USA formation somewhat. Addition of acetone powder extract did not show this effect.

The results fit the following interpretations: (1) The first step of USA synthesis is present in the acetone powder and is heat labile. (2) The second step is contained in mitochondrial extract and is more heat stable (although a considerable part of the activity is also destroyed by heating at 54° for 3 minutes). (3) The second step seems to be limiting in the untreated mitochondrial extract, since addition of the heated mitochondrial enzyme increased USA formation, while addition of acetone powder extract had no effect.

Table 3. Involvement of several enzymes in ureidosuccinate synthesis. Substrate and conditions as in Table 1.

Enzyme preparation	μmole of ureidosuccinate formed
Mitochondrial extract (ME), 1 mg enzyme-N	1.45
Mitochondrial extract, 3 min. at 54° (MEH) (1.5 mg enzyme-N)	0.10
Acetone powder extract (AP), 0.5 mg enzyme-N	0.07
ME + MEH	1.90
ME + AP	1.38
MEH + AP	0.42

Table 4. Stepwise synthesis of ureidosuccinate.

	Experiment No.													
	1		2		3		4		5		6		7	
	st. 1	st. 2	st. 1	st. 2	st. 1	st. 2	st. 1	st. 2	st. 1	st. 2	st. 1	st. 2	st. 1	st. 2
Substrate 1							+		+			+		+
Substrate 2								+		+	+		+	
Substrate 1+2	+		+		+									
Enzyme source:														
Acetone powder extract (0.5 mg N)	+		+				+			+	+			+
Mitochondrial extract, 3 min. at 54° (2.0 mg N)	+				+			+	+			+	+	
μmole ureidosuccinate formed	0.16		0.02		0.05		0.21		0.06		0.05		0.00	

Substrate 1: 30 μmole $^{15}NH_4Cl$, 20 μmole $NaHCO_3$, 10 μmole ATP, 50 μmole PGA, 15 μmole acetylglutamate, 25 μmole $MgCl_2$, 100 μmole phosphate buffer, pH 7.2, 2 mg MP. Volume = 3 ml.
Substrate 2: 30 μmole L-aspartate-^{15}N, 100 μmole phosphate buffer, pH 7.2. Volume = 3 ml. 60 minutes incubation at 37° for each step.

A more direct demonstration of this reaction sequence is given by the experiments summarized in Table 4. Experiments 1—3 demonstrate again that the presence of both the heated mitochondrial enzyme and the acetone powder is required for USA synthesis. In experiments 4—7 the study of USA formation was carried out in stepwise sequence. The substrate was divided into two parts: substrate 1 containing everything necessary for reaction (1), *i.e.* the formation of compound X; substrate 2 containing only aspartate (and buffer). In the first step an enzyme preparation was incubated with one substrate for 60 minutes at 37°. The reaction was then stopped by addition of 0.5 ml *N* PCA to the cooled solution. After 5 minutes in an ice bath the solution was neutralized to pH 7 with *N* $NaHCO_3$. The second step was then studied by the addition of the other enzyme and substrate, and incubation for an additional 60 minutes at 37°. Using the four possible combinations of substrates and enzymes it could be shown that the first step required the enzyme from the acetone powder extract and the substrate necessary for the formation of compound X, while the second step involved the participation of aspartate and the enzyme from the heated mitochondrial extract.

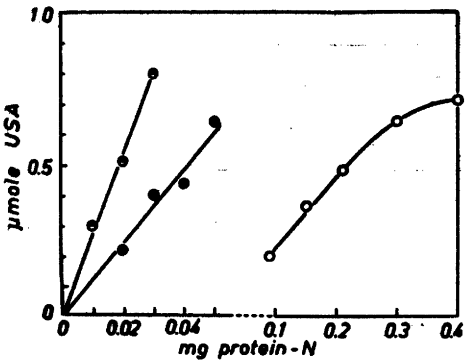


Fig. 4. Formation of ureidosuccinate by enzyme from E. coli. ○ = *unfractionated extract,* ● = *protamine fraction,* ◒ = *ammoniumsulfate fraction. The abscissa gives the amount of enzyme nitrogen per experiment for each point. Incubation at 37°, 15 min.*
Substrate: 10 µmole L-*aspartate-*^{14}C*, 2.6 µmole compound X, 100 µmole phosphate buffer, pH 6.5. Volume 1.3 ml.*

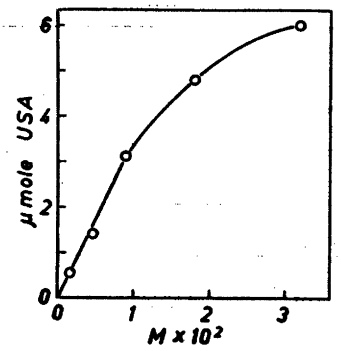


Fig. 5. Dependence of USA synthesis on L-*aspartate.*
Substrate: 7.7 µmole compound X, 150 µmole phosphate buffer, pH = 6.5, and L-*aspartate-*^{14}C *as indicated on abscissa. Volume 3.7 ml. Incubation 15 min. at 37° with 0.07 mg enzyme-N (ammonium sulfate fraction).*

A direct demonstration of reaction (3) was obtained by incubation of mitochondrial enzyme with aspartic acid and compound X. In this experiment L-aspartate-^{14}C was used. The resulting time curve of USA formation is shown in curve II of Fig. 2.

Experiments with enzyme from *E. coli.*

Studies of the condensing enzyme from liver which catalyzes reaction (3) were greatly hampered by the instability of the enzyme. The enzyme was also found to be present in rat kidney, spleen and heart, yeast autolysate and an extract of lyophilized *E. coli*. By far the highest activity was observed in the bacterial extract and further investigations were therefore carried out with the enzyme from this source.

Assay system. The bacterial extract showed no ability to synthesize USA from ammonia, bicarbonate and aspartic acid and thus contained little or no enzyme activity for reaction (1). Since the bacterial enzyme formed no compound X, it was not necessary to use purified compound X for the assay. It was instead prepared in solution by incubation of ammonia, $NaHCO_3$, ATP and acetylglutamate together with "enzyme B" [8], as described in the experimental part. The reaction was stopped by addition of 0.2 volumes of 0.5 *M* PCA to the cooled solution. After centrifugation and neutralization to pH 6.5 with *N* KOH an aliquot of the solution containing 2.6 µmole of compound X was added to 10 µmole of aspartate-^{14}C and 100 µmole of phosphate buffer pH 6.5. The bacterial enzyme was added and incubation (final volume 1.3 ml) carried

out for 15 minutes at 37°. Under these conditions USA formation (in the range 0.2—0.5 μmole) was proportional to the amount of enzyme added (see Fig. 4).

Quite large amounts of substrate are needed to saturate the enzyme (see below) and the conditions described do not permit maximal USA synthesis. Lack of sufficient substrate did not allow work with substrate-saturated enzymes. When an exact assay of the enzyme activity was desired several experiments at different enzyme concentrations were carried out and satisfactory results were obtained.

Purification of the enzyme. The lyophilized *E. coli* preparation was obtained through the courtesy of Dr. G. Hedén of the Bacteriological Department of the Karolinska Institutet. The cells had been grown on a synthetic liquid medium [12] for 18 hours. After this time the cells were harvested in a Sharples centrifuge, washed once with 0.9 % NaCl and lyophilized. At this stage the preparation, when stored in a desiccator in the cold room, retained enzymatic activity for at least one year.

Extraction of the cells was carried out by sonic vibration with glass beads (0.28 mm diameter). Five g of the lyophilized powder was homogenized in 50 ml of 0.1 *M* $NaHCO_3$. 40 ml of glass beads were added and vibration carried out by a "microid flask shaker" (Griffin and Tatlock, Ltd) at room temperature for 90 minutes. The very viscous solution was separated from the glass beads by filtration. The beads were washed with 50 ml of 0.1 *M* $NaHCO_3$. The combined filtrate and washings were centrifuged at 20 000 g at 20° for 30 minutes. 65 ml of slightly turbid supernatant was obtained.

To the supernatant was added 32.5 ml of a 2 % solution of protamine sulfate (neutralized to pH 8 with 0.5 *N* NaOH). The resulting stringy precipitate was centrifuged and the supernatant discarded. The precipitate was finely homogenized in 15 ml of 0.1 *M* potassium phosphate buffer, pH 7. After centrifugation the precipitate was once more extracted in the same way and centrifuged. All operations up to this step were carried out at room temperature.

The combined supernatants (30 ml) were cooled in an ice bath. Solid ammonium sulfate (6.35 g) was added to bring the solution to 30 % saturation. After standing for 10 minutes the precipitate was centrifuged off and discarded. More ammonium sulfate (3.1 g) was added to the supernatant and the solution centrifuged after 10 minutes. The resulting precipitate contained most of the enzymatic activity. It was dissolved in 5 ml of 0.1 *M* phosphate buffer, pH 6.5, and could be stored in the deep freeze. Most experiments were carried out

Table 5. Purification of condensing enzyme from E. coli.

	mg protein nitrogen	total units	units / mg N
Extract	259	1 220	4.7
Protamine precipitate	37	870	23
Ammonium sulfate, 30—45 % sat.	11.5	620	54

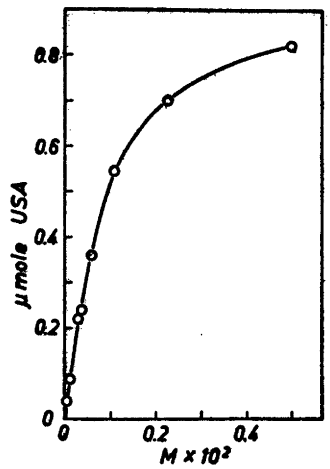


Fig. 6. Dependence of USA synthesis on compound X. Substrate: 5 µmole L-aspartate-^{14}C, 150 µmole phosphate buffer, pH 6.5, compound X as indicated on abscissa. Volume 3.7 ml. Incubation at 37° for 15 min. with 0.07 mg enzyme nitrogen (ammonium sulfate fraction).

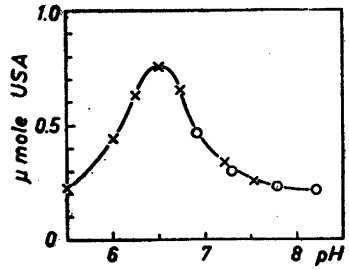


Fig. 7. pH curve of USA synthesis. Substrate: 4.5 µmole compound X, 5 µmole L-aspartate-^{14}C, 250 µmole buffer (× = phosphate, ○ = glycylglycine). Incubation at 37° for 15 min. with 0.07 mg enzyme nitrogen (ammonium sulfate fraction). Volume 3.7 ml.

with this fraction. Dialysis of the solution for 18 hours at + 2° against 0.01 *M* phosphate buffer resulted in the loss of ca 30 % of the activity. The dialyzed solution could be lyophilized without further loss of activity. A summary of the purification procedure is given in Table 5.

Some properties of the reaction. The dependence of the reaction on the simultaneous presence of L-aspartate and compound X is demonstrated by Figs. 5 and 6. All experiments subsequently described were carried out with purified compound X. Although it was not possible to effect enzyme saturation under the present experimental conditions, the results are sufficient to indicate that high concentrations of compound X and especially aspartate would be necessary for optimal synthesis*. The pH optimum of the reaction was at 6.5. Phosphate was without influence on the reaction (Fig. 7).

Mg^{++}, Zn^{++}, Mn^{++}, Ca^{++} and Fe^{++} did not stimulate USA formation by lyophilized, dialyzed bacterial enzyme under the following experimental conditions. In each experiment 1.0 mg of enzyme was incubated with 5 µmole of L-aspartate-^{14}C and 4 µmole compound X in a final volume of 2.8 ml, pH 7.2. In the presence of 10 µmole of either $MnCl_2$, $ZnCl_2$, $CaCl_2$, or $FeSO_4$ the following amounts of USA (in µmoles) were synthesized during 30 minutes at 37°: 1.11; 1.00; 1.09; 1.09. In the control experiment without addition of divalent ions, 1.01 µmole of USA was formed. In another similar experiment 0.81 µmole

* The experiments were complicated by the fact that at high concentrations of the reactants a non-enzymatic formation of USA took place. This was negligible under the conditions for enzyme assay. However, under the conditions of Fig. 5 an increasing part of the USA synthesis observed at aspartate concentrations above 0.5×10^{-2} *M* was non-enzymatic.

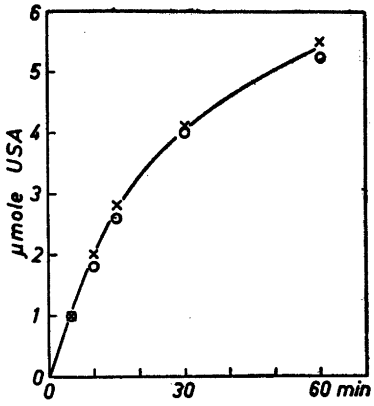
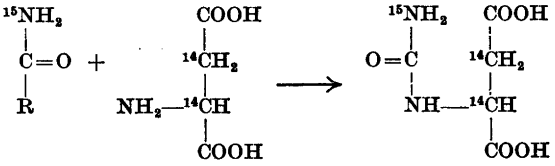


Fig. 8. Time curve and stoichiometry of USA formation.
Substrate: 25 µmole L-aspartate-^{14}C, 22 µmole compound X-^{15}N, 1 250 µmole glycyl-glycine buffer, pH 7.0. × = values calc. from ^{14}C; o = values calc. from ^{15}N. Volyme = 15 ml. For further explanation see text.

of USA was formed in the presence of 30 µmole of $MgSO_4$, while 0.85 µmole was formed in the control.

Stoichiometry of the reaction. The formation of one molecule of USA per one molecule of aspartate and compound X each was demonstrated by the simultaneous use of ^{15}N-labeled compound X and L-aspartate-^{14}C in one experiment. The USA formed stoichiometrically under these experimental conditions should be doubly labeled according to:



compound X + aspartate → ureidosuccinate

Compound X was enzymatically synthesized from $^{15}NH_4Cl$ (32 % excess ^{15}N). The carbamino group of USA therefore contained the same excess of ^{15}N. The succinate moiety of USA contained the same specific activity of ^{14}C as the precursor aspartate. The experiment summarized in Fig. 8 demonstrates the stoichiometric relation. The figure represents a time curve of USA formation. At each time point the amount USA formed was calculated independently from both the ^{15}N and the ^{14}C, respectively, of the reisolated USA. Within experimental error identical values were obtained for each time point.

DISCUSSION

The experiments described here demonstrate the occurrence in rat organs and *E. coli* of an enzymatic reaction by which ureidosuccinic acid, an intermediate in pyrimidine biogenesis, is formed from aspartic acid and compound X. Compound X has earlier been described as an intermediate in citrulline synthesis by Grisolia and Cohen [8]. The formation of compound X from CO_2,

NH_3, ATP and a substituted glutamate derivative seems therefore to represent one common step for both citrulline and USA biosynthesis. It is interesting that we were not able to demonstrate the occurrence of this enzymatic reaction in the bacterial extract. The possibility exists that the enzyme responsible for compound X formation had been destroyed during the preparation of the extract. On the other hand it was not possible to demonstrate this reaction in homogenates from most rat organs, which contained the enzyme forming USA from compound X. In the rat, with the exception of liver, only kidney preparations were found to form compound X, and here only minimal synthesis was obtained.

It seems unlikely that liver and possibly kidney are the only mammalian organs capable of synthesizing pyrimidines. The widespread existence of the enzyme capable of forming USA from compound X and aspartate also argues against this. The possibility arises, therefore, that compound X is synthesized by another mechanism than reaction (1). An example for such a reaction is the recently demonstrated conversion of citrulline-ureido-^{14}C to labeled USA, which probably proceeds *via* compound X as an intermediate [13].

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Short Communications

The Occurrence of Free Ornithine and its N-Acetyl Derivative in Plants

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When investigating the free amino acids in plants it was found in this laboratory that *Asplenium* species surprisingly contained many unknown ninhydrin-positive substances. Virtanen *et al.*[1,2] have recently isolated α-aminopimelic acid, γ-hydroxy-α-aminopimelic acid and its lactone from *Asplenium septentrionale*. We noticed that on hydrolysis of the free amino acid fraction of *Aspl. nidus*, even with 1 *N* HCl a large amount of ornithine was formed while the unknown spot 72 disappeared entirely (Fig. 1). A smaller amount of free ornithine was also present before hydrolysis. This was identified both chromatographically and by vanillin[3] spraying.

Ornithine is known to be a part of some cyclic peptides, tyrocidin[4], gramicidin[5] and bacitracin[6]. The free amino acid has only twice been chromatographically identified in plants[7,8]. Manske[9] found, however, in 1937, that the roots of *Corydalis ochotensis* contained 10 % of mono-N-acetylornithine based on the weight of air-dried material. We therefore examined the Finnish specimen *Corydalis bulbosa* and found a spot on the chromatograms, which was identical with that of our unknown substance. We have now been able to isolate this substance from the leaves of *Asplenium nidus*.

The 70 % ethanol extract (380 g fresh wt., 55 g dry wt.) was first purified with Amberlite IR-120 resin. However, only the basic amino acids, arginine, ornithine and lysine remained on the column. This was presumably due to the high content of slimy material, which filled the column, because the second treatment with the same resin gave only a mixture of neutral and acidic amino acids. Acidic amino acids were removed with Amberlite IR-4 B resin and the remaining solution of neutral amino acids was then fractionated by means of Dowex 50 resin (column 26 × 520 mm). 100 fractions of 20 ml each with 1.5 *N* HCl and 120 fractions af 15 ml each with 2.5 *N* HCl were taken. Fractions 25—60 contained serine and threonine, 40—45 glycin and alanine, 40—60 the lactone of γ-hydroxy-α-aminopimelic acid, 60—65 valine *etc.* and 160—190 only the unknown amino acid in question. The eluate was evaporated *in vacuo* at 33° C to a syrup (126.2 mg). After drying in a vacuum desiccator a crystalline mass (105.4 mg) was obtained on the addition of a small amount of absolute ethanol. It was found chromatographically that during concentration 3—5 % of the compound was decomposed to ornithine, but crystallization from ethanol-ether gave 20.8 mg of pure product. The substance decomposed at 200° C. (Found: C 34.62; H 6.54. Calc. for $C_7H_{14}N_2O_3 \cdot 2$ HCl: C 34.02; H 6.53. On titration 0.919 mg required 0.80 ml 0.00917 *N* NaOH in water and 1.19 ml in 80 % ethanolic solution. Found equivalent weights 125.14 and 84.13. Calc. for $C_7H_{14}N_2O_3 \cdot 2$ HCl: 123.57 and 82.38, respectively). By the method of Virtanen and Pulkki[10] 6.22 mg acetic acid was found from 26.2 mg substance (97.0 % of theor.). Acetate was qualitatively identified by means of $FeCl_3$, iodine and $La(NO_3)_3$, by As_2O_3 as cacodyl oxide and dry distillation with $Ca(CO_2)_2$ to acetone, which reacts with *o*-nitrobenzaldehyde to form indigo.

The unknown amino compound was chromatographically identical with acetylornithine isolated by Manske. It was unstable in the presence of nitrogen oxides (from $NaNO_2$ and HCl) and gave negative results with *p*-dimethyl-aminobenzaldehyde, isatin, iodine, vanillin and $KMnO_4$ when the

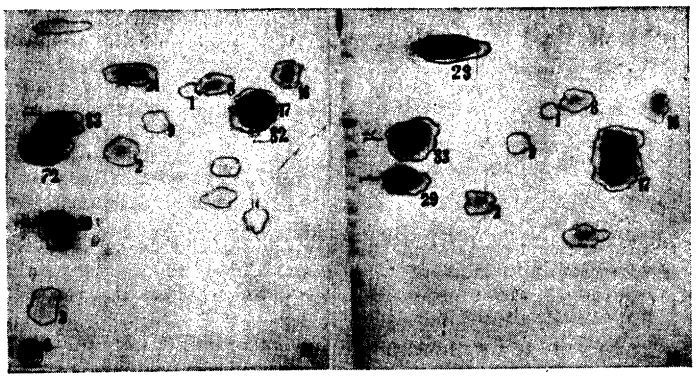

Fig. 1. Two-dimensional paper chromatograms of purified 70 % ethanol extract containing free neutral and acidic amino acids of Asplenium nidus; before hydrolysis (left) and after hydrolysis (right). 1 = gly, 2 = ala, 3 = val, 6 = phen.ala, 8 = ser, 9 = threo, 16 = asp, 17 = glu, 23 = orn, 24 = glutamine, 29 = γ-aminobutyric, 72 = acetylorn, 82 = γ-hydroxy-α-aminopim, 83 = lactone of 82.

reagents were sprayed on the chromatograms. Negative results using the methods of Hanes and Isherwood [11] and Chargaff, Levine and Green [12] indicated that the substance did not contain any phosphorus or sulphur. On paper electrophoresis it proved to be neutral, but after hydrolysis strongly basic ornithine appeared. From all these data it is clear that the substance in question is mono-N-acetyl-ornithine.

No chromatographical data about this substance are to be found in the literature. We obtained the following R_F-values:

	Butanol-acetic acid-water	Phenol-NH_3-water
Proline	0.32	0.91
Alanine	0.27	0.62
Acetylornithine	0.28	0.84
Ornithine	0.07	

In the protein hydrolysate no ornithine could be found. In addition to *Aspl. nidus* and *Corydalis bulbosa*, *Aspl. septentrionale*, *Aspl. viviparum* and *Aspl. trichomanes* also contained acetylornithine. *Dryopteris linnaeana*, *D. filix mas*, *Woodsia ilvensis*, *Eupteris aquilina*, *Struthiopteris filicastrum* and *Athyrium filix femina* were investigated, but no acetylornithine could be found.

Acetylornithine-N in *Aspl. nidus* was about 10 % of the free amino acid nitrogen and 1.14 % of the total nitrogen.

We wish to thank professor A. Kalela for *Asplenium* species obtained from the University of Helsinki Botanical Gardens and Dr. H. F. Manske for a sample of acetylornithine.

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On the Content of Hexosamines in Cell Nuclei of Calf Thymus and Calf Liver

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In 1951 Bychkov *et al.*[1] claimed that mucopolysaccharides and mucoproteins are regular constituents of the cell nuclei. They based this conclusion on hexosamine determinations made on dried tissues and on nuclear preparations.

However, the method of Elson and Morgan [2] used by them is known to give too high values in the presence of basic amino acids and neutral sugars [3]. This combination is present in the cell nuclei, where basic proteins and pentoses from the nucleic acids make up a considerable part of the dry matter.

The application of a chromatographic step [4] before applying the Elson and Morgan reaction offers a possibility for the determination of hexosamines without any interference of foreign substances.

Bychkov *et al.*[1] used dilute citric acid for the isolation of the cell nuclei [5]. Data presented by Mirsky *et al.*[6] indicate that dilute citric acid may remove from 18 to 55 % of the proteins of the nucleus as compared with the results obtained in using anhydrous lipid solvents for the isolation of the nuclei. It thus seemed necessary to reinvestigate the possible content of hexosamines in cell nuclei.

We used the method of Mirsky *et al.*[6] for the isolation of cell nuclei from calf thymus and calf liver. The final purity of the preparations was judged by staining with crystal violet.

The hexosamine content was determined according to Gardell [4] on hydrochloric acid hydrolysates of the preparations.

From calf thymus we obtained 4.38 g of nuclei from 100 g of dried tissue. Mirsky *et al.*[6] obtained 20—25 g/100 g. Our preparation looked very pure on microscopical examination, and seemed to be practically free from cytoplasmic contamination.

The liver preparation, on the contrary, was not so pure. Our yield of 7.9 g of nuclei out of 200 g of dried tissue is even more than double the yield of Mirsky *et al.*[6]

The analytical results are shown in Table 1.

The figures for the hexosamine content in whole thymus tissue are derived from six chromatographic runs on three different hydrolysates. In five of them two peaks were obtained, one for glucosamine, and one for galactosamine. The galactosamine peak contained some 10—15 % of the total hexosamine content, the glucosamine peak the remaining 85—90 %. The sixth chromatogram showed only a glucosamine peak.

The figures for the hexosamine content in thymus cell nuclei are also derived from six chromatographic runs on three different hydrolysates. Only in three of them there was a trace of galactosamine.

The same results were obtained in analyzing another preparation of cell nuclei from calf thymus.

As the purity test of the nuclear preparations is entirely subjective, one cannot draw extensive conclusions from our results. We cannot exclude the possibility that the amount of hexosamines in our nuclear preparations is due to cytoplasmic contamination. One can state anyhow, that the amount of hexosamine found by Bychkov *et al.*[1] is at least five times too large. If the cell nuclei really contain hexosamines, the amount is of the order of

Table 1.

Percentage of	Thymus		Liver	
	Whole tissue	Nuclei	Whole tissue	Nuclei
Nitrogen (micro-Kjeldahl)	15.65	16.60	10.40	10.87
Ash	12.20	12.2	4.98	5.06
Moisture	5.1	7.1	7.1	6.8
Hexosamine	0.24	0.09	0.23	0.13

magnitude of 0.1 % of the dry substance or less. Mucopolysaccharides containing galactosamine are not present in the cell nuclei of calf thymus.

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Uptake of ^{35}S-labelled Sulfate in the Heparin of a Dog Mastocytoma

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Oliver, Bloom and Mangieri [1] in 1947 found dog mast cell tumors to be very rich in heparin. The heparin content of a highly differentiated tumor was some fifty times that of the dog liver. This finding has been confirmed by Riley and co-workers [2] in 1954. In 1953 Jorpes, Odeblad and Boström [3], by means of an autoradiographic technique, observed that ^{35}S-labelled sulfate is taken up by the mast cells in the subcutaneous tissue of the rat. They could not, however, determine whether the exchange observed occurred in heparin, in any of its precursors or in chondroitin sulfuric acid.

It was therefore considered of interest to know if the sulfate groups of heparin can be labelled with ^{35}S. The large content of heparin in mast cell tumors makes them a suitable material for the study of this question. At the suggestion of Dr. B. Åberg the following experiment was therefore performed.

Experimental. To a dog suffering from a rather highly differentiated mastocytoma was given 1 mC of carrier free sodium sulfate intravenously. After 24 hours the tumor, weighing 16.7 g, was removed and frozen to —20° C. After mincing in the Latapie mill, the material was digested for three weeks with proteolytic enzymes according to the technique of Gardell [4]. After digestion the suspension was treated with several volumes of 96 % ethanol at pH 8.8. The precipitate formed was suspended in water at pH 8—9 and after centrifugation the solution was passed through an anion exchange column, Dowex 2, (Boström and Månsson [5]) in order to remove inorganic sulfate. In a control test with a mixture of heparin and radioactive sulfate it was found that the radioactivity was retained, while the heparin passed through.

The sulfate free solution was evaporated *in vacuo* to dryness and dissolved in water to a concentration of 5 %. The pH was adjusted to 7—8. Then 0.2 volumes of 20 % barium chloride were added. The precipitate was separated by centrifugation. The supernatant was precipitated by adding a third of its volume of glacial acetic acid. The precipitate weighed 3.060 mg. To the mother liquor three volumes of 96 % ethanol were added. This precipitate weighed 9.795 mg. The former precipitate should contain heparin and the latter chondroitin sulfuric acid or the heparin monosulfuric acid found by Jorpes, Werner and Åberg [6] to occur in the normal mast cells. These compounds can easily be separated by

Table 1.

	Weight in mg	Counts/min./cm² of 3 mg	I. U. of heparin / mg	
			Whole blood method	Thrombin method
Barium chloride-acetic acid precipitate	3.060	6 690	61	96
Ethanol precipitate from the mother liquor	9.795	1 750	8.5	12

magnitude of 0.1 % of the dry substance or less. Mucopolysaccharides containing galactosamine are not present in the cell nuclei of calf thymus.

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It was therefore considered of interest to know if the sulfate groups of heparin can be labelled with ^{35}S. The large content of heparin in mast cell tumors makes them a suitable material for the study of this question. At the suggestion of Dr. B. Åberg the following experiment was therefore performed.

Experimental. To a dog suffering from a rather highly differentiated mastocytoma was given 1 mC of carrier free sodium sulfate intravenously. After 24 hours the tumor, weighing 16.7 g, was removed and frozen to —20° C. After mincing in the Latapie mill, the material was digested for three weeks with proteolytic enzymes according to the technique of Gardell [4]. After digestion the suspension was treated with several volumes of 96 % ethanol at pH 8.8. The precipitate formed was suspended in water at pH 8—9 and after centrifugation the solution was passed through an anion exchange column, Dowex 2, (Boström and Månsson [5]) in order to remove inorganic sulfate. In a control test with a mixture of heparin and radioactive sulfate it was found that the radioactivity was retained, while the heparin passed through.

The sulfate free solution was evaporated *in vacuo* to dryness and dissolved in water to a concentration of 5 %. The pH was adjusted to 7—8. Then 0.2 volumes of 20 % barium chloride were added. The precipitate was separated by centrifugation. The supernatant was precipitated by adding a third of its volume of glacial acetic acid. The precipitate weighed 3.060 mg. To the mother liquor three volumes of 96 % ethanol were added. This precipitate weighed 9.795 mg. The former precipitate should contain heparin and the latter chondroitin sulfuric acid or the heparin monosulfuric acid found by Jorpes, Werner and Åberg [6] to occur in the normal mast cells. These compounds can easily be separated by

Table 1.

	Weight in mg	Counts/min./cm² of 3 mg	I. U. of heparin / mg	
			Whole blood method	Thrombin method
Barium chloride-acetic acid precipitate	3.060	6 690	61	96
Ethanol precipitate from the mother liquor	9.795	1 750	8.5	12

means of the solubility of their barium salts (Jorpes and Gardell [7]).

The anticoagulant activity was assayed by the whole blood method of Jalling, Jorpes and Lindén [8] and by a thrombin titration method described by Quick [9]. The radioactivity was measured in a Geiger-Müller counter. The condition of infinite thickness of the preparation is not reached for the 0.1 MeV beta-radiation of ^{35}S until it has a thickness of some 30 mg/cm^2. As this condition could not be fulfilled with our preparation, we decided to measure the activities of 3 mg/cm^2. For that reason the counts of the two preparations are not quite comparable.

The amino sugar content of the two precipitates was determined according to Gardell [10]. Results in Table 1.

As is evident from the table a highly active barium salt of heparin was obtained. In accordance herewith it was found that the substance contained 13.2 % of glucosamine and 0.45 % of galactosamine. The ethanol precipitate contained 3.35 % glucosamine and 2.17 % galactosamine. It is not conceivable that anything but heparin could account for the radioactivity of the barium chloride — acetic acid precipitate. Consequently this infers that sulfuric acid groups of heparin can be labelled with ^{35}S.

This investigation was aided by a grant to B. Åberg and B. Larsson from *The Swedish Cancer Society*.

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Note on the Rheology of Polyacrylonitrile Solutions

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Suspension polymers of acrylonitrile, prepared by the *azo* activator technique [1], show apparent deviations from the ordinary behaviour of *redox* (emulsion or solution) polymers that calls for further attention. As earlier pointed out [1] suspension polymers do not dissolve at room temperature in dimethyl formamide even in a very broad range of molecular weight. At said temperature redox polymers may show complete solubility, and solutions containing more than 15 % by weight of polymer can ordinarily be prepared. Even if the different end groups in the two types of polymers may be one reason for this anomaly, it seems to be an undue simplification to ascribe the effect entirely to the interaction between solvent and terminating groups. The final step in the kinetic scheme of polymerization is generally shared between chain coupling and disproportionation [2], which would mean that other end groups than those derived from the activator would be present.

Some peculiarities, connected with suspension polymerization of acrylonitrile, should be shortly emphasized. Even at low degrees of conversion the monomer droplets are converted into a porous (spongy) particle. This polymer is insoluble in its monomer, and a two-phase system is obtained, where the reaction mainly proceeds on the large interphase. The supply of monomer at the reaction centres is secured by the action of capillary forces and by diffusion. As the particle grows acrylonitrile is consumed, why the propagation rate gradually decreases. The reaction ceases asymptotically by deficit of monomer and evidently shows a fairly long step gradually retarded due to a decreasing supply of acrylonitrile. Just as in the case of polyvinyl chloride [3] an excessive branching, chain transfer and an abnormal molecular size distribution should be expected at said reaction step. If sufficient activator is present, it is permitted to ask if even a slight crosslinking takes

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means of the solubility of their barium salts (Jorpes and Gardell [7]).

The anticoagulant activity was assayed by the whole blood method of Jalling, Jorpes and Lindén [8] and by a thrombin titration method described by Quick [9]. The radioactivity was measured in a Geiger-Müller counter. The condition of infinite thickness of the preparation is not reached for the 0.1 MeV beta-radiation of ^{35}S until it has a thickness of some 30 mg/cm^2. As this condition could not be fulfilled with our preparation, we decided to measure the activities of 3 mg/cm^2. For that reason the counts of the two preparations are not quite comparable.

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place by an initiating action on traces of bifunctional impurities (*e. g.* divinyl acetylene) generally present in the technical grade of acrylonitrile, prepared from acetylene.

The mentioned features of the polymerization mechanism ought to influence on the physical properties of the polymer in general, but particularly on its behaviour in solution. Even low degrees of branching and crosslinking act as steric hindrances, causing deviations from the *random flight* type of molecular configuration in dilute solutions. A look upon the general theory on the properties of macromolecular substances in solution, developed by Kirkwood [4,5], Riseman [4], and Flory [6], seems to be appropriate here. The intrinsic viscosity, $[\eta]$, of a polymer is stated to be a linear function of the effective volume of the separate molecular coils in dilute solution. Thus, if $\bar{r}^2$ is the mean-square end-to-end distance of the coil, the following relation applies:

$$[\eta] = \Phi \cdot (\bar{r}^2)^{3/2}/M \qquad (1)$$

where M is a measure of the molecular weight of the polymer, Φ a universal parameter (approximative value $2 \cdot 10^{21}$, r beeing expressed in cm and $[\eta]$ in decilitres per gram), Apparently $(\bar{r}^2)^{3/2}$ indicates the effective volume of the molecular coil in solution. In the actual case it is preferably split into its component factors, referring to the unpertubated mean end-to-end distance r_0 and the coefficient of deformation α. This gives the key equations

$$[\eta] = \Phi \cdot (\bar{r}_0^2/M)^{3/2} \cdot M^{\frac{1}{2}} \cdot \alpha^3 \qquad (2)$$

Table 1. Viscosity gradient of a polymer of acrylonitrile, prepared in a redox system. Activator: ammonia persulphate-sodium sulphite. Average visc. mol. weight = 57 000. Solid content of solution: 18.0 %.

Temp., °C	Viscosity, sec. (average from 4 measurements)
50	94.2
70	55.6
90	34.2
110	21.6
130	14.4
Activation energy of flow, cals. mole^{-1}	6.1×10^3

or

$$[\eta] = K \cdot M^{\frac{1}{2}} \cdot \alpha^3 \qquad (3)$$

where $K = \Phi \cdot (\bar{r}_0^2/M)^{3/2}$. The magnitudes K and α are in general dependent of the temperature. By differentiating the logarithms of eq. (3) we obtain

$$\mathrm{d}\ln [\eta]/\mathrm{d}T = 3\,\mathrm{d}\ln \alpha/\mathrm{d}T + \mathrm{d}\ln K/\mathrm{d}T \qquad (4)$$

which reveals that the intrinsic viscosity gradient is mainly determined by the change of the factor of expansion α with temperature. If steric hindrances are present, we should expect an abnormal increase in the value of α, as all effects implying hindrances to free rotation expand the chain to a larger configuration [6]. The

Table 2. Viscosity gradients of acrylic polymers, prepared in suspension systems. Solid contents of solutions: 18.0 %.

Polymer	1	2	3	4	5	6	7	8
Aver. visc. mol. weight. 10^{-3}	51	54	55	59	70	75	44	45
Temp., °C	← Viscosity in seconds →							
50	120	136	244	249	171	320	64	87
70	69	90	127	137	110	158	34	58
90	38	49	77	90	59	106	18	34
110	19	28	42	57	36	71	11	17
130	15	19	26	34	23	45	8	10
Act. energy, cals. mole$^{-1} \times 10^{-3}$	6.8	7.2	7.3	6.5	6.5	6.4	6.8	7.0

corresponding influence on the magnitude $[\eta]$ was earlier implicitely formulated $[\eta] = K'M^a$, where the exponent is to regard as a comprehensive parameter, including deviations from "ideal" behaviour. Only at the *Flory point* [7], Θ °K, intramolecular effects disappear and the relation (3) reduces to $[\eta]\Theta = KM^{\frac{1}{2}}$, a formula showing a large similarity to the preceding expression.

What has been said about the intrinsic viscosity should also be valid in a qualitative sense for viscosity at higher concentrations as far as the dependence of the volume expansion factor is concerned. At a fixed concentration of the polymer in a good solvent, the viscosity gradient (or its computed equivalent, the activation energy of flow) is expected to show different values for various types of polymers where irregularities in structure cause deviations from the standard state (straight addition chains).

In accordance with the considerations above a series of suspension polymers was prepared and their viscosity-temperature gradients measured in the range 50—130° C. The activation energy of flow was computed from the Andrade [8] formula

$$\eta = Ae^{E/RT} \qquad (7)$$

giving the value of E as the slope of the straight line

$$2.3 \log \eta = \log A + E/R \cdot (1/T)$$

where the viscosity value may be expressed in arbitrary units. The explicit value of the activation energy is preferably written

$$E = 4.6\ \Delta \log \eta/\Delta 1/T \text{ cals.mole}^{-1} \qquad (8)$$

Experimental. Eight polymers were prepared in accordance with the method earlier described [1], using azo-*bis* (*iso*butyronitrile) as an activator and tertiary dodecylmercaptan as a modifier *. The average viscosity molecular weight was varied in the desired range ($44—75 \cdot 10^3$) by using different amounts of the modifier. Solutions of the polymers were prepared by wetting the granules at 20° C with the required amount of dimethyl formamide for at least ten minutes. On slow agitation the slurry was gradually heated to 80° C, where dissolution is complete within 60 minutes. Dilute solutions can be prepared already at 40—45° C, but practical difficulties (high viscosity, slow rate of swelling) makes dissolution at higher temperatures more attractive in the actual case. A concentration of 18.0 % was chosen. This value seems roughly to be comparable with the value 17.8 %, used in a recent investigation [9], as the suspension polymer contains residues of activator and modifier, which are more difficult to remove by washing than water soluble catalysts. In the cited study [9] an activation energy of flow of 5 700 cals.mole^{-1} was found, measurements beeing made in the *Jullander* consistometer at small angles of shear. In the present case some direct reference measurements were carried out on a *redox* (emulsion) polymer, synthesised in the laboratory. The general features of redox polymerization are described elsewhere [1,3]; the most important point in this connection is to observe that the reaction is terminated (interrupted) as soon as the amount of monomer present is insufficient to maintain saturation in the aqueous phase.

The viscosity gradients of the redox polymer and the suspension polymers are found in Tables 1 and 2, respectively. All measurements of viscosity were carried out in test tubes of 200 mm measuring range (diametre 20 mm, falling ball method). A lot of 15 balls (stainless steel, diametre 2.0 mm) were selected from a larger assortment and calibrated in a filtered dope of polyacrylonitrile at room temperature. Heating of the test tubes was arranged in a thin, light lubricating oil and the temperature measured at the centrum line of the tubes.

* In the present investigation acrylonitrile from acetylene was used throughout all polymerizations. Average content of divinyl acetylene: 260 p. p. m.

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Sedimentation Studies of Cytochrome c

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Pedersen [1] more than 15 years ago determined the sedimentation constant ($S°_{20} = 1.83 \times 10^{-13}$ *s*) and the diffusion coefficient ($D°_{20} = 11.3 \times 10^{-7}$ $cm^2 \cdot s^{-1}$) of the purified cytochrome c obtained by Theorell and Åkeson [2] from beef heart. Recently, Atlas *et al.*[3] reported that they had determined corresponding data of cytochrome c from different sources. Their values have been quoted by Edsall [4] as $S°_{20}$ about 2.3×10^{-13} *s* and $D°_{20}$ about 13×10^{-7} $cm^2 \times s^{-1}$ for cytochrome c from beef, horse and pig. Because of these discrepancies and the modified preparation methods developed [5,6], we found that a more systematic investigation would be of interest.

The sedimentation studies were made with a Spinco analytical ultracentrifuge model E, using a synthetic boundary cell, which is especially suited for work on slowly sedimenting molecules. The reliability of the method was controlled by running bovine albumin (Armour fraction V) both in the common and the synthetic boundary cell. This substance has previously been used in a comparative study of values obtained by a Spinco centrifuge and an oil turbin centrifuge [7]. The values thus obtained agreed fully with these data with a discrepancy less than 1 %. All our runs on cytochrome preparations were made at 260 000 g, and 0.05 *M* phosphate buffer of pH 6.8 with 1 % sodium chloride added was consequently used. Protein concentrations between 1 and 5 mg/ml were used, and in none of the cases could any concentration dependence of $S°_{20}$ be seen.

In a few cases we tried to determine the diffusion coefficient at 23°, using a Tiselius apparatus with the Philpot-Svensson optical system, and a cell 15 mm long and 2 mm broad. An interference filter (Bausch & Lomb) after the lamp allowed a red light of 639 mμ so that the image on the plate was homogeneously illuminated. Because of the low illumination, exposures of several minutes were necessary. The diffusion coefficients were calculated according to the "height-area" method. The percentage of error of our data appeared to be as high as ±10 %, mostly due to an imperfect initial boundary and disturbances during the compensation procedure in the narrow cell.

For salmon and chicken cytochrome the apparent partial specific volume was determined both in water and in the buffer, by means of a common pycnometer. No significant influence of the buffer could be

Table 1.

Material	Preparation method	% Fe	$S°_{20} \times 10^{13}$	Runs in the centrifuge	$D°_{20} \times 10^{7}$	V_p	f/f_0
Beef	P &N [5]	0.42	1.64±0.01	4	(11.3) [1]	(0.707) [2]	1.25
Horse	P &N	0.41	1.89±0.15	2	9.5	(0.707)	1.34
Horse	L &B [6]	0.31	1.61±0.06	2			1.41
Horse	L &B	0.36	1.61±0.02	2			1.41
Horse	L &B	0.36	1.55	1			1.43
Salmon	P &N *	0.41	2.33±0.15	3	10.2	(0.707)	1.19
Salmon	P &N	0.45	2.19±0.15	3			1.22
Salmon	P &N	0.48	1.76±0.10	6			1.31
Salmon	L &B	0.27	1.50±0.02	6	10.7	0.75	1.25
Salmon	L &B	0.27	1.45±0.10	3			1.26
Chicken	P &N	0.37	1.63±0.03	8	(11.3)	0.72	1.23

* Some denaturation might have occurred in this preparation.

seen. Our results are summarized in Table 1. The errors given for $S°_{20}$ are the calculated standard deviations of the mean of each preparation.

It is seen that $S°_{20}$ varies both between the different species and between the two preparation methods for the same species. In spite of their lower iron content, the preparations according to Loftfield and Bonnichsen give lower $S°_{20}$ values than those according to Paléus and Neilands. This indicates that the former preparations might contain impurities with slightly lower sedimentation constant than cytochrome c itself. The two components cannot be resolved but should give rise to a higher apparent diffusion constant which, however, could not be detected in the ultracentrifuge. The high value of V_p for salmon cytochrome c according to Loftfield and Bonnichsen might also be due to this impurity.

In only two cases we got sedimentation constants comparable to those of Atlas *et al.*[3], but one of them might have been modified by partly denaturation.

Our value for beef cytochrome c is 10 % lower than the cited value of Pedersen. This is in accord with the known difference of 5 to 10 % between the oil turbin and the Spinco centrifuge [7, 8].

The calculated frictional ratios also agree quite well with the older value 1.29 of beef cytochrome but not with the data of Atlas *et al.* close to 1.0. The rather high values of f/f_0 for horse cytochrome c might reflect that the diffusion constant of 9.5×10^{-7} cm^2 $\times$ s^{-1} is somewhat too low.

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Effects of X-rays and Water Content on Sugars in Barley Seeds

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An investigation of the influence of ionizing radiations on biochemical systems demonstrated that the relative concentrations of simple sugars in growing *Vicia faba* plants were strongly affected by chronic γ-irradiation [1]. When resting (*i. e.*, ripe) barley seeds are irradiated, their radiosensitivity was found to decrease with increasing water content [2]. Since the concentration of reducing sugars has been shown [3,4] to increase with increasing moisture content, we found it important to investigate possible relations between sugar content and radiation sensitivity. Under certain conditions glucose may act as a protective agent [5].

Methods. Seeds of the two-rowed barley strain, Bonus, were treated for 6 days with streaming air of the relative humidities 0 % and 90 %. This treatment gave samples containing 7.5 % and 17 % H_2O, respectively. The seeds were irradiated with 175 kV unfiltered X-rays at an intensity of 4 000 r min.$^{-1}$. Analysis of reducing sugars was made 15 hours after irradiation, and again after 2—4 weeks' storage at a constant water content and at 20° C.

For the analysis the material was ground twice in a Wiley mill, using first a 20-mesh, then a 40-mesh sieve. The grist was boiled for 3 min. in 80% ethanol, and thereafter extracted, with the same solvent, for 3 hours in a micro-Soxhlet apparatus. (Using 96 % ethanol instead, only about two thirds of the sugars were extracted by the same procedure.) After deproteinizing [6], reducing sugars were titrated, with an accuracy of 2 %, according to Somogyi [7]. The maximum variation between individual extractions amounted to 5 %.

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Results. It was demonstrated (Table 1) that in the unirradiated material the in-

Table 1. Content of reducing sugars (mg glucose/g dry weight) after irradiation and storage at different water contents.

Water content %	Analysis after	Seeds irradiated with, r		
		0	5×10^5	10×10^5
7.5	15 hours	1.46	1.53	1.46
	4 weeks	1.51	1.61	1.64
17	15 hours	1.15	1.33	1.21
	4 weeks	1.92	2.21	2.05
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crease of the water content first caused a fall in reducing sugars, possibly a consequence of the increased respiration [3, 4]. During 2—4 weeks storage, at unchanged water content, the value increases, possibly becoming slightly higher at the higher water content.

Irradiation with 5×10^5 r increases the content of reducing sugars, especially at the higher water content. (10×10^5 r gives a somewhat lesser chemical change. After treatment with this high dose, a fraction of the seeds do not germinate; and death of seeds might therefore be responsible for any disturbed values.) As this increase is already observed immediately after irradiation, it is probably not an effect of a changed metabolic rate (*e. g.*, decrease of respiration rate [9]). It might be ascribed to a radiation induced disintegration of, *e. g.*, starch. If this is so, intermediates originating from cellular constituents other than carbohydrates and water, obviously play a role, as the disintegration of starch, irradiated at 8—23 % H_2O, was only measurable as viscosity decrease.

It can be concluded that irradiation causes a greater chemical change in sugars when the seeds have a higher moisture content, and consequently are protected against biological damage. The significance of this result, for the interpretation of the mechanism of action of radiations, will be discussed elsewhere [10].

1. Jaarma, M. *Suomen Kemistilehti* **B 28** (1955) 40.
2. Lefort, M. and Ehrenberg, L. *Arkiv Botan.* [2] **3** (1955) 121; Ehrenberg, L. *Botan. Notiser* **108** (1955) 184.
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The States ^1I of Trivalent Praseodymium and Thulium

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The identification by Spedding [1] of the states of Pr^{+3} has for many years been assumed to be complete. But the proposal of Ellis [2] that the band in the orange is due to a transition to $^1D_2$ rather than to $^1I_6$ was recently supported by the crystal field studies of Hellwege [3]. The Condon-Shortley theory [4] was applied to this f^2-system by Trefftz [5]. This author calculated the electrostatic interaction parameters F_2, F_4, and F_6 for a hydrogen-like 4f-wave function with an effective charge $Z_0 = 9.48$ (this seems very reasonable, because the ionization energy of Ce^{+3} corresponds to $Z_0 = 6.56$). However, the value of F_6 calculated by Trefftz is 64 times too small due to a misinterpretation of Condon and Shortley's decimal point as a multiplication operator. This led Trefftz to accept Spedding's very small value of F_6.

In the author's opinion*, F_6 is not necessarily negligible [6-8]. The position of ^1D, relatively to ^3P, can easily be accounted for by the set of ratios [6] $F_4 = 0.2\,F_2$ and $F_6 = \frac{1}{7}\,F_2$, given in Ref. 8, p. 19.

* *Note added in proof:* Judd [15] has recently drawn the same conclusion from Eu^{+3}. The present author must here correct the highest value among the three diagonal elements of energy of the ^5D-states to $9E^1 - 11E^3$ rather than $9E^1 + \frac{143}{7}\,E^3$,

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0.02 F_2 just as well as by Trefftz's values, $F_4 = 0.138\ F_2$ and $F_6 = 0.0152\ F_2$ (if F_6 multiplied by 64). Also Racah's values [9] for Th^{++} suggest a similar decrease in the F^k integrals: $F_4 = 0.19\ F_2$ and $F_6 = 0.018\ F_2$. Both Trefftz's revised and the other proposals give ^1I at nearly the same place as the triplet ^3P. Thus, it will probably be masked by the three strong bands in the blue. Broer, Gorter and Hoogschagen, in their investigation of band intensities [10], could not explain the anomalously high intensity of the ^1I-band of Pr^{+3}. Hellwege's identification of the band as $^1D_2$ removes this difficulty, and one must consider the ^1I as not yet observed. The triplet character [10] expressed in squares of the wave function will only be of the magnitude [1].

$$[S = 1] = \frac{6\,\zeta^2}{(^1I_6 - {}^3H_6)^2}$$

Since $\zeta = 400$ cm^{-1} and the distance $^1I_6 - {}^3H_6$ between the two perturbing levels is $\sim 15\,000$ cm^{-1}, the quantity $[S = 1]$ will only be ~ 0.005. Even if the molar extinction coefficient ε_0 of the band $^3H_4 - {}^1I_6$ were 20 for the pure triplet transition, the actual ε_0 would only be 0.1. Nevertheless, this should be observable. The author has tried in vain in the wavelength range 550—270 mμ for 0.2 M $Pr(ClO_4)_3$ in 5 cm cells. However, the spectrum does show a conspicuous broadening towards shorter wavelengths of the band $^3P_1$, but no definite conclusion can be drawn.

According to Pauli's equivalence theorem of holes, f^{12} in Tm^{+3} and f^2 in Pr^{+3} have the same set of terms. The band at 350 mμ of Tm^{+3}, supposed to be due to $^3H_6 - {}^1I_6$, is cited above [10] as the only other known example of anomalously high intensity. But an aqueous solution of thulium(III) perchlorate in 1 M $HClO_4$ shows this band as not having any larger oscillator strength P (which is proportional to the area of the band as a function of wave number [10]) than the other bands, while Hoogschagen, Scholte and Kruyer [11] give $P \sim 30 \cdot 10^{-6}$, contrary to usual lanthanide bands with $P \sim 10^{-6}$. The materials used by these authors seem to contain strongly ultraviolet-absorbing impurities (iron?) as was true in the case of their holmium [11]. Measurements on a Cary spectrophotometer of 0.1 M $Tm(ClO_4)_3$ in 2 cm cells give the following maximum values ε_0 and oscillator strengths P (uncertainty $\sim$20 %):

Maxima observed	ε_0	P	P (Ref. [11])
780 mμ	1.0	$1.9 \cdot 10^{-6}$	$1.98 \cdot 10^{-6}$
695,682,655	0.9; 1.5; 0.3	3.0	3.18
462	0.4	0.4	$\sim$0.45
358,355	0.8; 0.8	2.4	$\sim$30
290,284	0.1; 0.2	0.4	
276,274	0.1; 0.2	0.3	
263,260	1.4; 1.2	5	

Qualitative agreement is found with the measurements by Rodden [12] for wavelengths over 350 mμ. Besides the bands of the table, weak and narrow bands at 520, 378 and 255 mμ (with $\varepsilon_0 \sim 0.1$) may be ascribed to an impurity of $\sim$ 3 % erbium, whose strong bands occur at these wavelengths [8]. Below 300 mμ the background rises appreciably, and below 230 mμ a steep absorption limit appears. The latter

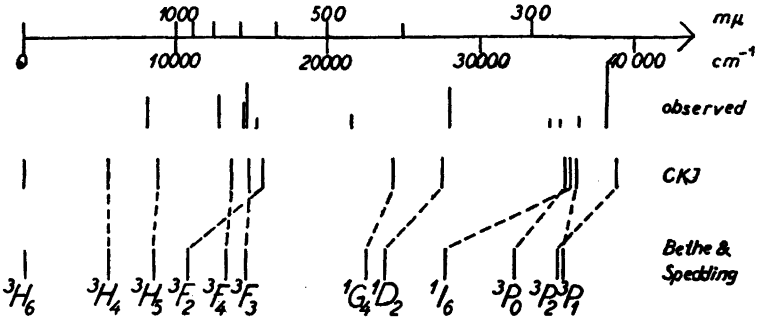

Fig. 1. Observed bands of Tm^{+3} (except that at 8 100 cm^{-1}) represented by peaks proportional to the oscillator strength P. Calculated positions with intermediate coupling from the parameters, given in the text.

is not due to ClO_4^- and is perhaps due to $4f^{13}-4f^{11}5d$ transitions in Tm^{+3}. The three band groups in the range 290–260 mμ are equivalent to those found by Gobrecht [13] in solid $Tm_2(SO_4)_3$, $8H_2O$. The multiplet ^3P is somewhat perturbed, the most intense band group 263–260 mμ presumably being due to $^3H_6-{}^3P_2$.

Since $[S = 1] = 0.02$ for $^1I_6$ in Tm^{+3}, the transition to this state is still of unusually high probability, if it corresponds to the 358–355 mμ band group. This is not certain, since the strong intermixing of the states with $J = 4$, *viz.* $^3H_4$, $^3F_4$ and $^1G_4$, may distribute two of the states to 685 and 355 mμ. Bethe and Spedding [1,14] choose the set of parameters $F_2 = 308$ cm^{-1}, $F_4 = 71$ cm^{-1}, and $F_6 = 0.86$ cm^{-1} by extrapolation from [3] La^+. If the possibility [6] $F_2 = 450$ cm^{-1}, $F_4 = 90$ cm^{-1}, and $F_6 = 9$ cm^{-1} is applied to Tm^{+3} with $\zeta = -1\,400$ cm^{-1}, the identifications are changed somewhat. Fig. 1 gives the two sets of calculated wave numbers and the observed. But the question cannot be settled, before J can be determined from crystal field studies. The low intensity of the band at 462 mμ might support the identification with $^1D_2$.

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*iso*Thiocyanates XI.

4-Methylthiobutyl *iso*Thiocyanate, a New Naturally Occurring Mustard Oil

ANDERS KJÆR and ROLF GMELIN

Chemical Laboratory of the University of Copenhagen, Denmark *

In a previous communication of this series [1] it was observed that seeds of *Eruca sativa* Mill. contain a volatile mustard oil which is paperchromatographically different from the known, naturally occurring *iso*thiocyanates. We now wish to report on the identity of this mustard oil as 4-methylthiobutyl *iso*thiocyanate (I), a compound first synthesized by Schmid and Karrer [2].

$$CH_3SCH_2CH_2CH_2CH_2-N=C=S$$

I

$$CH_3SCH_2CH_2CH_2CH_2-NHCSNH-R$$

II R = H

III R = $CH_2C_6H_5$

From a larger seed sample, made available to us during the summer of 1954, we isolated the thiourea derivative (II) of the volatile *iso*thiocyanate. The chemical structure was established by analysis and by comparison with a synthetic specimen of (II). Mixed melting point, paper chromatography in various solvents and infrared spectra left no doubt as to the identity of the two preparations.

In Nature (I) occurs as a yet uncharacterized glycoside for which we suggest the name *glucoerucin*. Its presence is disclosed upon paper chromatography according to Schultz and Gmelin [3]. These authors noticed in seeds of *Eruca sativa*

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two glucosides one of which was interpreted as belonging to their "SO-group" whereas the higher spot was attributed to glucotropaeolin. Upon paper chromatography of our crude thiourea we could exclude the presence of N-benzylthiourea. Consequently, the spot of presumed glucotropaeolin undoubtedly represented glucoerucin, the two being hardly distinguishable in the usual glucoside chromatography.

In older literature seeds of *Eruca sativa* are reported to contain an unidentified mustard oil [4] whereas Mohammad and Ahmad [5] tacitly assumed allyl *iso*thiocyanate to be present in the seeds, known in India as "taramira" and of importance for the local oil production (jamba oil). Sudborough *et al.*[6] isolated and purified the volatile mustard oil from *Eruca sativa* seeds and transformed it into a derivative, m. p. 83—84°, upon reaction with benzylamine. The composition $C_{19}H_{25}N_3S_3$ was reported for this derivative, apparently without suggestions as to its chemical structure. Therefore, we submitted authentic 4-methylthiobutyl *iso*thiocyanate to reaction with benzylamine and obtained in good yield the expected N-(4-methylthiobutyl)-N′-benzylthiourea (III), $C_{13}H_{20}N_2S_2$, with m. p. 77—78°. We are inclined to believe that the Indian authors have been mislead by inaccurate analyses inasmuch as the calculated figures for the two formulae do not deviate very much (see Experimental).

The characteristic radish-like smell and taste of (I) proved of much diagnostic value in our search for the new mustard oil in various species. The taste, in conjunction with paperchromatographic results, points to (I) as being widely distributed in the plant kingdom. So far, we consider 4-methylthiobutyl *iso*thiocyanate to be present in seeds of *Iberis sempervirens* L., *Vesicaria utriculata* L. and *V. sinuata* Poir. *Matthiola annua* R.Br. and various species of *Cheiranthus* and *Brassica oleracea* L., as well as root materials of *Hesperis matronalis* L. and *Farsetia clypeata* R.Br. seem to contain (I) or closely related *iso*thiocyanates.

It is pertinent here to refer to two previously recorded, naturally occurring *iso*thiocyanates with the same carbon skeleton as that of (I). From seeds of *Erysimum Perofskianum* Fisch. et M., Schneider and Kaufmann [7] isolated erysolin (IV), representing the sulphone corresponding to (I).

$$CH_3SO_2CH_2CH_2CH_2CH_2-N=C=S$$

IV

$$CH_3SOCH=CHCH_2CH_2-N=C=S$$

V

Sulphoraphen, the characteristic *iso*thiocyanate of radish seed (*Raphanus sativus* L.), was shown by Schmid and Karrer [8] to possess the structure (V), differing from (I) in being unsaturated and containing a sulphoxide-grouping. It is interesting in this connection that Lavine [9] demonstrated the intermediate formation of a "dehydro"-derivative in the oxidation of methionine to its sulphoxide.

The isolation and characterization of further *iso*thiocyanates, structurally related to (I), will form the subject of future communications.

Experimental. Isolation and structure proof of (II). A preliminary argentometric analysis of the air-dried, fatty seeds of *Eruca sativa* Mill. showed a content of 1.4 % of volatile mustard oil, calculated as methylthiobutyl *iso*thiocyanate.

A homogenized mixture of 25 g of seeds of *Eruca sativa* (fat-containing) and 500 ml of water was placed at 35° for 3 hours. The volatile constituents were removed by steam distillation, 500 ml of distillate being collected and transformed into thiourea upon standing for 24 hours with 100 ml of conc. ammonia. Evaporation *in vacuo* left a brownish, crystalline residue which was taken up in hot water, treated with a little charcoal, filtered and cooled. Colourless, nacreous plates separated and were recrystallized twice from water and once from ethyl acetate and petroleum ether before analysis. The yield was 0.25 g, m. p. 53° (Fisher-John's block), undepressed on admixture with an authentic specimen of N-(4-methylthiobutyl)-thiourea (II). (Found: C 40.71; H 7.87; N 15.62. Calc. for $C_6H_{14}N_2S_2$: C 40.42; H 7.91; N 15.72). Paper chromatography with chloroform as the solvent [10] gave the R_{Ph}-value 0.97, whereas in the following systems: 1) pyridine : amyl alcohol : water (35:35:30); 2) heptane : 90 % formic acid: *n*-butanol (20:20:15)[12]; 3) heptane : 90% formic acid : *n*-butanol (20:10:22)[12], R_F-values of 0.82, 0.13 and 0.76 were found, respectively, for both the natural and the synthetic specimen. The infra-red spectra were determined in potassium bromide discs and proved to be completely identical.

Synthesis of (I) and (II). 4-Methylthiobutylamine (6.3 g), prepared by our previously

recorded method [11], was dissolved in water (45 ml), and a solution of thiocarbonyl chloride (6.1 g) in chloroform (50 ml) was added. To the vigorously shaken mixture 1.4 *N* NaOH (76 ml) was slowly added. After 0.5 hour, the chloroform layer was separated, dried and evaporated. Distillation *in vacuo* of the residue afforded 5.1 g (60 %) of the *iso*thiocyanate, b.p. 136° at 12 mm, with a characteristic smell of radish. Ref.[3] lists b. p. 130—140° at 9 mm.

The mustard oil was dissolved in ethanol, previously saturated with ammonia at 0°, and left at room temperature for 3 days. The solvent was removed and the residue recrystallized twice from water and once from chloroform and petroleum ether before analysis. M. p. 53° (Fisher-John's block). Ref.[3] reports m. p. 55—56°. (Found: N 15.80; S 36.07. Calc. for $C_6H_{14}N_2S_2$: N 15.72; S 35.96).

N-(4-Methylthiobutyl) - N' - benzylthiourea (III). A solution of (I) (615 mg) and benzylamine (735 mg) in ethanol (3 ml) was kept at 40° for 18 hours. The solvent was then removed and the residue taken up in ether. The solution was shaken with small portions of 0.5 *N* HCl and water in order to remove the excess amine. During these operations the separation of the crystalline thiourea started (900 mg). Before analysis, the sample was recrystallized twice from 70 % ethanol and once from ethyl acetate and petroleum ether, m. p. 77—78° (water bath). (Found: C 58.25; H 7.55; N 10.45. Calc. for $C_{13}H_{20}N_2S_2$: C 58.15; H 7.51; N 10.44. Calc. for $C_{19}H_{25}N_3S_3$: C 58.28; H 6.44; N 10.81).

Microanalyses by Mr. W. Egger. We wish to thank the *Botanical Garden of Copenhagen* for cultivating *Eruca sativa* Mill. for our use. Thanks are also due to Dr. Nuding of the *Landwirtschaftl. Hochschule, Stuttgart-Hohenheim* and Mr. R. von Hösslin of the *Staatl. Lehr- und Forschungsanstalt für Gartenbau, Weihenstephan b. München*, for furnishing us with further samples of the same seeds.

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The Calculation of Sedimentation Velocity from Experiments with Concentrated Mixtures

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A sedimenting boundary in the ultracentrifuge is by no means mathematically simple even in the case of a dilute solution of an ideal solute, as is seen from Faxéns [1] solution of the concentration function in a centrifuge cell. Additional complications, to which considerable attention has been paid, arise from thermodynamic and frictional effects, and from polydispersity. Thus the situation may be very complicated and is still more accentuated if the solution is concentrated *in a more hydrodynamic sense* [2], in which case the general form of the differential equation describing the course of the concentration change in the cell is intricate, even apart from effects of the non-constancy of the thermodynamical and frictional coefficients.

In such a situation it may sometimes seem more natural to use the concentration decrease in the middle of the sectorshaped cell than to use an analytical property of the boundary. This well-known possibility is evident from the considerations of Rinde [3, 4], of Trautman and Schumaker [5] and others.

In a part of the cell, not reached by the diffusion gradient regions at the top and bottom, the determination of the actual sedimentation coefficients of a two-com-

recorded method [11], was dissolved in water (45 ml), and a solution of thiocarbonyl chloride (6.1 g) in chloroform (50 ml) was added. To the vigorously shaken mixture 1.4 *N* NaOH (76 ml) was slowly added. After 0.5 hour, the chloroform layer was separated, dried and evaporated. Distillation *in vacuo* of the residue afforded 5.1 g (60 %) of the *iso*thiocyanate, b.p. 136° at 12 mm, with a characteristic smell of radish. Ref.[3] lists b. p. 130—140° at 9 mm.

The mustard oil was dissolved in ethanol, previously saturated with ammonia at 0°, and left at room temperature for 3 days. The solvent was removed and the residue recrystallized twice from water and once from chloroform and petroleum ether before analysis. M. p. 53° (Fisher-John's block). Ref.[3] reports m. p. 55—56°. (Found: N 15.80; S 36.07. Calc. for $C_6H_{14}N_2S_2$: N 15.72; S 35.96).

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The Calculation of Sedimentation Velocity from Experiments with Concentrated Mixtures

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A sedimenting boundary in the ultracentrifuge is by no means mathematically simple even in the case of a dilute solution of an ideal solute, as is seen from Faxéns [1] solution of the concentration function in a centrifuge cell. Additional complications, to which considerable attention has been paid, arise from thermodynamic and frictional effects, and from polydispersity. Thus the situation may be very complicated and is still more accentuated if the solution is concentrated *in a more hydrodynamic sense* [2], in which case the general form of the differential equation describing the course of the concentration change in the cell is intricate, even apart from effects of the non-constancy of the thermodynamical and frictional coefficients.

In such a situation it may sometimes seem more natural to use the concentration decrease in the middle of the sectorshaped cell than to use an analytical property of the boundary. This well-known possibility is evident from the considerations of Rinde [3, 4], of Trautman and Schumaker [5] and others.

In a part of the cell, not reached by the diffusion gradient regions at the top and bottom, the determination of the actual sedimentation coefficients of a two-com-

ponent system is quite simple even in the cases of the most complicated boundaries indicated above.

Disregarding the diffusion term, the two-component case is described by (*cf.* Ref.[2], eq. (11); V is the partial specific volume)

$$\frac{\partial m_2}{\partial t} = -\frac{1}{x}\frac{\partial}{\partial x}\left(\frac{m_1^2 m_2^2 (V_1 - V_2) V_1 \omega^2 x^2}{\varphi_{12}}\right) \quad (1)$$

Rinde showed for this non-diffusion case that the concentration decrease during the process is uniform over the cell and is thus the same at different levels. This statement is a consequence of the condition of continuity and does not assume a dilute system. However, it assumes that we start from a constant composition in the cell. Such being the case, we have the advantage that the concentrations m_1, m_2 g/cm³ and the friction per cm³ φ_{12} are independent of x, which reduces equation (1) to

$$\frac{\partial m_2}{\partial t} = -2m_1^2 m_2^2 (V_1 - V_2) V_1 \omega^2 / \varphi_{12} \quad (2)$$

The sedimentation coefficient of component 2 is [2]

$$s_2 = m_1^2 m_2 (V_1 - V_2) V_1 / \varphi_{12} \quad (3)$$

which, by substituting equation (2), can be consequently determined from

$$s_2 = -\frac{1}{2m_2\omega^2}\frac{\partial m_2}{\partial t} \quad (4)$$

by an experimental determination of $\partial \ln m_2/\partial t$ somewhere in the middle of the cell.

Equation (4) is the same here as in the theory of the sedimentation of a dilute substance. As already mentioned, the equation assumes a uniform concentration over the cell. We will stick to this restriction but will drop now the restriction to two components. For the ith component, the continuity equation for the "cylindrical" flow in the cell of sector shape is

$$\frac{\partial m_i}{\partial t} = -\frac{1}{x}\frac{\partial}{\partial x}(s_i m_i \omega^2 x^2) = -2s_i m_i \omega^2 \quad (5)$$

where $s_i\,\omega^2 x$ is, by definition, the sedimentation velocity in cm/sec. For any partial quantity, in this case the partial volume V_i, $\Sigma V_i dm_i = 0$. Combined with equation (5) this gives an equation previously proved for two components

$$\Sigma s_i m_i V_i = 0 \quad (6)$$

The deduction shows that this special relation is not restricted to constant partial volumes, as distinct from the special relations which connect sedimentation coefficients with frictions, which were reported elsewhere [2].

It is often more practical to measure the change of refractive index n. Applying the approximation introduced previously [2] in writing the refractive index n of the mixture

$$n = n_1^\circ m_1 V_1^\circ + n_2^\circ m_2 V_2^\circ \quad (7)$$

(where the index ° refers to the pure component) and, in addition, assuming $V_1 = V_1^\circ$ and $V_2 = V_2^\circ$ as in the case of an ideal mixture, s_2 may be calculated according to

$$s_2 = \frac{\partial n/\partial t}{2(n_1^\circ - n)\omega^2} \quad (8)$$

n_1° is the refractive index of the medium, n that of the solution as measured in the cell during the experiment. The general expression $m_1 V_1 + m_2 V_2 = 1$ has been used in the deduction. As symmetry has been preserved, a corresponding approximate expression is valid also for s_1. Equations other than (7) are not considered here.

Owing to its simplicity, expression (8) seems to be worth notice even if more exact expressions may prove necessary in many instances. Several-component cases must be specially considered. When using this procedure of measuring s for low-molecular mixtures, much will depend on the control of temperature, the sensitivity being excellent in itself if interference methods of observation are employed.

Conclusions. Owing to the complicated nature of a sedimentation boundary, it may sometimes be of value to use the general concentration change in the middle of the cell, the theory of which remains simple also in experiments with concentration effects. Some remarks are made on this procedure, which possesses a practical applicability also for the non-dilute system, the theory of which was treated in an earlier communication without mentioning any mode of practical calculation.

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Eine praktische Vereinfachung bei der Skalenmethode zur Messung von Brechungsindexgradienten

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Bei der bisher benutzten Anordnung z.B. für Diffusionsmessung [1,2] müssen die Skalenstrichverschiebungen nicht über eine gleichförmige Skala sondern über die Linien des Skalenphotogrammes, d.h. über die verschobenen Linienlagen aufgetragen werden, damit die Brechungsindexgradienten als Funktion der Höhenkoordinaten in der Diffusionszelle richtig herauskommen sollen. Es ist dies nachteilig an und für sich, führt aber ausserdem eine Korrektur mit sich, die von der Art der einfachsten Benutzung einer Referenzskala herrührt. Diese letztere ist ein Skalenphotogramm, dass ohne Konzentrationsgradient aufgenommen wird (jedoch mit Beibehaltung der übrigen Bedingungen), und dessen Linienlagen bei Berechnung der Skalenstrichverschiebungen des Hauptphotogrammes als Referenzwerte dienen. Dieses Verfahren dient zur Eliminierung optischer Unvollkommenheiten besonders der Diffusionszelle. Es setzt voraus, dass Referenzlinie und Linie der Hauptaufnahme derselben Lage in der Zelle entsprechen, was bei der bisherigen Anordnung nur ungefähr zutrifft. Die dafür zu berechnende Korrektur [2] kann nun in einfacher Weise vermieden werden.

Die Vereinfachung besteht in einem Platzwechsel von Skala und photographischer Platte. Bei der gebräuchlichen Apparatur, wo die Zelle zwischen Skala und Objektiv steht, ist die abbildende Projektion geradlinig von der photographischen Platte bis zum Objektiv und weiter zu der Zelle (wo sie durch die Lichtkrümmung gestört wird). Deshalb korrespondieren die (lichtkrümmenden) Zellenlagen mit den Lagen auf der Platte, d.h. mit den durch die Krümmung verzerrten veränderlichen Lagen. Bei dem entgegengesetzten Verfahren herrscht Geradlinigheit von der Skala zum Objektiv und bis zu der Zelle. Dadurch werden die Zellenlagen durch die Originalskala geregelt. Der Vorteil liegt teils darin, dass letztere gleichförmig ist, teils darin, dass die Zellenlagen einmalig definiert und von der Lichtkrümmung oder Brechung in der Zelle zunächst unabhängig werden.

Diese Beobachtung ist allgemeiner Art und gilt damit auch bei den modifizierten Verfahren mit zweimaliger Abbildung, sei es dass diese durch die Raumverhältnisse bedingt ist wie bei gewissen Ultrazentrifugen, oder aus anderen Gründen durchgeführt wird, wie bei der von Svensson [3] vorgeschlagenen verkürzten Anordnung. Massgebend ist ob die Aperturblende (das Projektionszentrum), zwischen Zelle und Platte (die alte Anordnung) oder zwischen Skala und Zelle steht.

Die Rolle der Aperturblende wird besonders klar im Falle zweimaliger reeller Abbildung, wobei wir der Reihe nach Skala, erstes Objektiv, erstes Skalenbild, zweites Objektiv und schliesslich zweites Skalenbild haben, wo die photographische Schicht angebracht ist. Ob die Zelle zwischen Objektiv I und Skalenbild I ("negativer Skalenabstand"), oder zwischen letzterem und Objektiv II steht, ist für unser Problem zunächst gleichgültig. Um scharfe Linien zu erzielen muss, je nach der Steilheit der Gradienten in der Zelle, die Apertur mehr oder weniger klein gewählt werden. Wird die diesbezügliche Blende am Objektiv II angebracht, so liegt die alte Anordnung vor, während eine Anbringung am Objektiv I die neu vorgeschlagene Anordnung liefert.

Die Messgeräte zur Answertung der Skalendiagramme wie sie von Schumacher [4] und von Claesson [5] konstruiert und ausgeführt worden sind, werden durch die Umänderung nur unbedeutend berührt und jedenfalls nicht in unvorteilhafter Richtung.

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Spaltung von D,L-Histidin bei Papierchromatographie

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Seit 1951 wurde mehrfach über razemische Aminosäuren berichtet, die bei Papierchromatographie mit optisch inaktiven Lösungsmittelsystemen in optische Antipoden gespalten werden. Der Grund hierfür muss in einer Adsorption an der optisch aktiven Cellulose liegen, wobei die eine Antipode stärker adsorbiert wird als die andere.

Zu diesen Aminosäuren gehören verschiedene Dioxyphenylalanine [1,2]. Die Rolle der Hydroxylgruppen dieser Aminosäuren könnte in der Bildung von Wasserstoffbrücken zur Cellulose liegen; eine Gesetzmässigkeit in der Stellung dieser Hydroxylgruppen im Benzolkern kann noch nicht festgestellt werden. Bei 2,5-Dioxyphenylalanin ist gezeigt worden [1], dass die L-Form die kürzere Wanderungsstrecke hat, d.h. vom Papier stärker adsorbiert wird; ob bei den anderen spaltbaren Dioxyphenylalaninen dieses auch der Fall ist, ist nicht bekannt, da nur die Razemate untersucht wurden [1,2].

Closs und *Haug* [3] berichteten über die Spaltung von D,L-Tryptophan bei Papierchromatographie und zeigten, dass die L-Form am stärksten auf dem Papier festgehalten wird. Mehrfach wurde die papierchromatographische Spaltbarkeit von D,L-Kynurenin mitgeteilt [4,5]. Bei Kynurenin scheint die Wasserstoffbrücke zwischen der aromatischen Aminogruppe und der Carbonylgruppe für die Spaltung notwendig zu sein, da eine Veränderung der Carbonylgruppe zur CH(OH)- oder CH_2-Gruppe diese Spaltbarkeit aufhebt [6]. Kynurenin würde somit aus zwei kondensierten Ringen bestehen und Ähnlichkeit mit Tryptophan aufweisen; bei Kynurenin wird aber die D-Form am stärksten adsorbiert.

D- und L-Phenylalanin zeigen keinen Unterschied in den R_F-Werten [4]. Es war von Interesse zu untersuchen, wie sich ein Austausch des Benzolringes in Phenylalanin gegen einen heterocyclischen Ring auf die Spaltbarkeit auswirkte. Aus diesem Grunde wurden papierchromatographische Versuche mit D,L-, D- und L-Histidin-mono-hydrochlorid ausgeführt. Es zeigte sich, dass razemisches Histidin in optische Antipoden gespalten wird und die D-Form die kürzeste Strecke wandert.

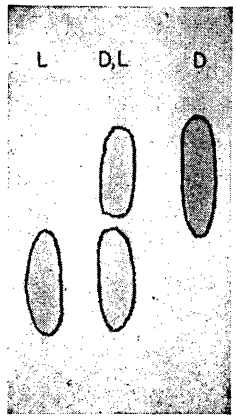

Abb. 1. Durchlaufchromatogramm von L-, D,L- *und* D-*Histidin-mono-hydrochlorid mit Methanol-Wasser (3:1) auf Whatman Nr. 4-Papier. Zeit: 23 ½ Std. Wanderungsstrecken der Antipoden:* D *26,3 cm,* L *29,7 cm. Wanderungsstrecken des gespaltenen Razemats:* D *26,2 cm,* L *29,7 cm.*

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Microestimation of Cyanide with a Modified Epstein Procedure

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For microestimation of cyanide the methods of Alridge [1] and Epstein [2] seem to be the most sensitive and accurate [3]. Both of them use a preliminary formation of cyanohalogenide, which is then reacting with pyridine and benzidine (Alridge) or phenyl-methyl-pyrazolone (Epstein), thus giving a coloured compound for colorimetry. For further increase of sensitivity two principles can be applied: A preliminary concentration of the sample by distillation or aeration [4] or concentration of the final coloured substance by extraction in *e. g.* butanol [5].

In our work with the hemiglobin-cyanide complex we had some experience with the Epstein procedure, which we modified by altering the preparation of reagents. In this way we increased the sensitivity 5 to 6 times, and the method became more convenient to handle. Using 4 ml of sample with a cyanide concentration from 10^{-5} *M* to 10^{-6} *M*, the cyanide content could be determined with a standard deviation of $\pm$ 3 %. The final extinction, read at 632 mμ, would vary from 1.0 to 0.1. Under these conditions Beers law is obeyed. The modification will be described here with a few comments.

Reagents. Chloramin-T, 0.5 % in water. Stable in the refrigerator for months. The concentration may vary from 0.25 to 1.0 %.

Pyridine. A. R. Stored in the refrigerator.

3-Methyl-1-phenyl-5-pyrazolone. Commercial product recrystallized to m. p. 127—128° C.

Bis-3-methyl-1-phenyl-5-pyrazolone. Can be prepared by boiling the monopyrazolone for several hours with phenylhydrazine but is more easily synthesized by gently refluxing 1 mole ethylacetoacetate with 3 mole phenylhydrazine for less than 0.5 h. The mixture will suddenly solidify. The solid bispyrazolone is boiled out three times with alcohol.

Mixed pyrazolone powder. A stock is made by mixing 5 parts mono- and 1 part bispyrazolone.

Pyridine-pyrazolone reagent. 150 mg mixed pyrazolone powder is dissolved in 25 ml pyridine. This reagent is stable for one week when kept in the refrigerator. The solution of bispyrazolone alone in pyridine [3] is only stable for 2 days. The ratios of mono- to bispyrazolone and of pyrazolone to pyridine are chosen so that the final colour will be maximal. Small variations will cause no influence on the final result, so the preparation of reagents can be made easily. The chosen amount of powder is easily dissolved in the cold pyridine.

Standard cyanide solution.

Procedure. A 4 ml sample, with pH between 4 and 8, is chilled. 0.2 ml chilled chloramine solution is added and mixed by swirling. After 5 minutes in an ice bath 0.8 ml chilled pyridine-pyrazolone reagent is added. The mixture is left at room temperature for 80-90 minutes. The blue colour is read in a colorimeter or in a spectrophotometer at 632 mμ. A known standard is always run simultanously.

5 minutes was found nesessary for quantitative chlorination. As the reagent was stored cold, it was preferred to add it chilled. It was found of no advantage to control temperature with a thermostate during colour development. The final colour was only little influenced by the temperature used. Using room temperature, maximum intensity of colour is obtained after 75 min. and the colour is stable for at least 0.5 h.

If the sample contains other substances than cyanide, these of course may interfere and make it necessary to run blinds.

Many attemps to increase the sensitivity further by previous aeration with the technique of Boxer and Rickards [4] and with modifications always failed to give satisfactory recoveries or reproducible results. On the other hand it was shown that extraction of the fully developed colour by butanol could increase the sensitivity at least 5 times, but as our experience with that method is only limited, a detailed description is omitted.

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Enzymatic Decarboxylation of γ-Hydroxyglutamic Acid to α-Hydroxy-γ-amino-*n*-butyric Acid

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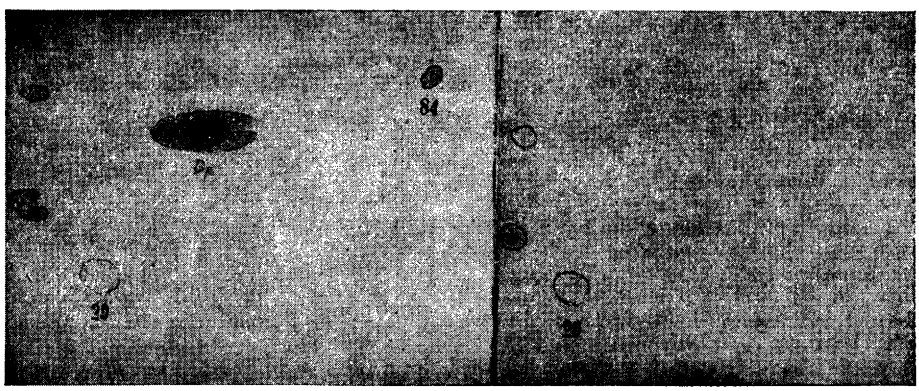
We have recently isolated γ-hydroxyglutamic acid from *Phlox decussata* [1]. Attempts to decarboxylate this acid with homogenates of *Phlox* led to negative results, whereas L-glutamic acid was easily decarboxylated with the same homogenates. Accordingly, *Phlox* did not contain the specific γ-hydroxyglutamic acid decarboxylase. The same result was obtained with homogenates of pea.

After fruitless experiments with green plants we found decarboxylase activity also with γ-hydroxyglutamic acid by using as enzyme material *Escherichia coli*, a B_{12}-vitamin requiring mutant. α-Hydroxy-γ-aminobutyric acid was hereby formed as the decarboxylation product (Fig. 1) which could be isolated and characterized (*cf.* below). This amino acid could not be detected in *E. coli* without addition of γ-hydroxyglutamic acid (70 % alcohol extract of bacteria, two-dimensional paper chromatography).

According to Umbreit and Heneage [2] *E. coli* decarboxylates *allo*-β-hydroxy-DL-glutamic acid (synth.). All strains of *E. coli* capable of decarboxylating this acid contained glutamic acid decarboxylase too, but the results suggested that the two enzymes are different. We have compared the velocities of the decarboxylation of L-glutamic acid, our γ-hydroxyglutamic acid, *allo*-β-hydroxy-DL-glutamic acid and β-hydroxy-DL-glutamic acid using *E. coli*. The CO_2 evolution was measured in a Warburg apparatus in N_2-atmosphere according to Schales *et al.*[3]. The results are presented in Table 1.

Isolation of the decarboxylation product. 27 mg of γ-hydroxyglutamic acid in 10 ml of 0.1 *M* acetate buffer (pH 4.9) + 1 g of fresh *E. coli* (178 mg dry subst.) was kept for 24 h at 38° C with occasional shaking. The bacteria were separated and washed with the buffer. The solution + washwater was passed through an Amberlite IR-120 column. The amino acids remained in the column and were eluted with 1 *N* ammonia. After evaporation *in vacuo*



a b

Fig. 1a. Two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3) of decarboxylation of α-amino-γ-hydroxyglutamic acid (84) by E. coli. Dp = decarboxylation product, 84 = traces of not decarboxylated γ-amino-α-hydroxyglutamic acid, 29 = γ-aminobutyric acid.

Fig. 1b. Control experiment without any substrate.

Table 1. Decarboxylation rates of hydroxyglutamic acids. Measurements were made with E. coli at pH 4.9 in a 0.1 M acetate buffer at 37° C and with Phlox and Pisum at pH 6.2 in a 0.1 M phosphate buffer at 30° C with a final substrate concentration of about 5.5 μmoles/ml in each case.

Agent	Q_{CO_2} (μl CO_2/hour/g dry weight)			
	L-Glutamic acid	γ-Hydroxy-glutamic acid	Allo-β-hydroxy-DL-glutamic acid	β-Hydroxy-DL-glutamic acid
E. coli	34 900	3 400	8 200	650
Phlox decussata	730	0		
Pisum sativum	3 000	0		

the solid rest was dissolved in 1 ml of phenol-water-solvent (500:184) and fractionated in a cellulose powder column (1.2 × 37 cm) on the top of which 1 mg methylorange was placed. 17 fractions of 1.5 ml were collected. The indicator came out in fractions 2—5, γ-aminobutyric acid in fractions 6—11, and the decarboxylation product of γ-hydroxyglutamic acid in fractions 11—17. The combined fractions 12—17 (9 ml) were diluted with alcohol and water, the amino acid separated in an Amberlite IR-120 column, eluted with ammonia and evaporated to dryness. After recrystallization from water the mp. was 199° C.

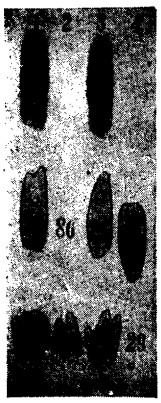


Fig. 2. Paper chromatogram of the pure decarboxylation product after reduction with HI and red P. 1 = reduction products + γ-aminobutyric acid (29), 2 = γ-aminobutyric acid alone, 3 = reduction products, 4 = decarboxylation product alone (86). Solvent phenol-NH_3. The slow moving spots are probably iodine-containing compounds.

Characterization of the decarboxylation product.

1. By reduction with HI (*d* 1.96) and red P at 140° C for 4 h. (1 mg substance + 1 mg P + 6 μl HI) γ-aminobutyric acid was formed (Fig. 2). Accordingly the decarboxylation product was a hydroxyderivative of γ-aminobutyric acid.

2. Analysis: N 11.55. Calc. for a monohydroxyaminobutyric acid N 11.75.

3. The decarboxylation product is not homoserine, and does not contain the α-amino group. Accordingly, the α-carboxyl group is split off by decarboxylation, and the decarboxylation product is α-hydroxy-γ-amino-*n*-butyric acid.

$$HOOC \cdot CHOH \cdot CH_2 \cdot CHNH_2 \cdot COOH \xrightarrow{-CO_2} HOOC \cdot CHOH \cdot CH_2 \cdot CH_2NH_2$$

Transamination: α-ketoglutaric acid + γ-hydroxyglutamic acid ⟶ glutamic acid + γ-hydroxy-α-ketoglutaric acid, could be found with homogenates of *Phlox*.

As far as we know α-hydroxy-γ-amino-*n*-butyric acid has not earlier been found in any biological material.

We are very grateful to Dr. Karl Pfister, Rahway, New Jersey, for preparations of synthetic β-hydroxy-DL-glutamic acid and *allo*-β-hydroxy-DL-glutamic acid.

1. Virtanen, A. I. and Hietala, P. K. *Acta Chem. Scand.* **9** (1955) 175.
2. Umbreit, W. W. and Heneage, P. *J. Biol. Chem.* **201** (1953) 15.
3. Schales, O., Mims, V. and Schales, S. S. *Arch. Biochem.* **10** (1946) 455.

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A New Type of Nitrogen Compound in Green Plants. A Cyclic Homoserine Derivative in Some *Liliaceae* Plants

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Species of *Convallaria* and related plants have been widely investigated because of the high content of many important alkaloids. We have, however, found no report in the literature confirming the free amino acid composition of these plants.

It was found by paper chromatography that *Polygonatum officinale* contained large amounts of an unknown ninhydrin-positive substance (I; spot 0 on Fig. 1). After ninhydrin spraying the color of the spot was brownish violet. A strong red color developed by treatment with sodium 1,2-naphthoquinone-4-sulfonate and alkali [1]. *p*-Dimethylaminobenzaldehyde gave a positive reaction, too, somewhat weaker only than with citrulline. When hydrolysed with 1 *N* HCl, substance I either disappeared totally or at least diminished very much and two other spots were formed. One of them (spot 51 on Fig. 2) had a typical violet color reaction with ninhydrin and was identified as homoserine. The other one (II; spot 00 on Fig. 2) appeared as a very intensive deep-yellow spot on chromatograms sprayed with ninhydrin. The color of this spot changed, however, rather rapidly to violet. Both I and II were quite stable against deamination with nitrogen oxides (from $NaNO_2$ and HCl). As will appear from the following, I is built up exclusively from homoserine though, in acid hydrolysis, it also gives substance II.

The unknown substance I was isolated by using Dowex 50 ion exchange resin, and HCl from the hydrochloride was removed with Amberlite IR-120. 4 g of a white crystalline product was obtained from 2.9 kg material (1.6 kg roots and 1.3 kg leaves; fresh wt.). Its elementary composition corresponded to the formula $C_4H_7O_2N$, hence the lactone of homoserine. (Found: C 47.22; H 6.83; N 13.94; equiv. wt. 101.9. Calc. for $C_4H_7O_2N$: C 47.51; H 6.98; N 13.87; Equiv. wt. 101.1). However, no lactone reaction could be detected. In the infrared spectra no typical absorptions either for lactones or for dioxopiperazines could be found. By

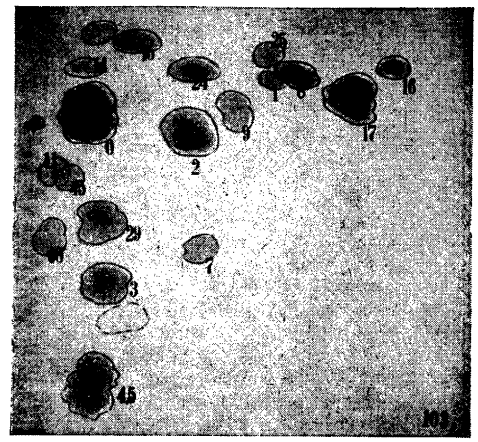


Fig. 1. Two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3) of the free amino acids of Polygonatum officinale. O = unknown I, 1 = gly, 2 = ala, 3 = val, 4 = ileu, 5 = leu, 7 = tyr, 8 = ser, 9 = threo, 11 = pro, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 24 = glu-NH_2, 25 = asp-NH_2, 29 = γ-NH_2-butyric, 45 = ethanolamine, 60 = piperidine-2-carboxylic acid.

Fig. 2. Partial hydrolysis (with HCl) of the unknown I. Alanine is added to the chromatogram. 00 = unknown II, 51 = homoserine, 81 = lactone of homoserine.

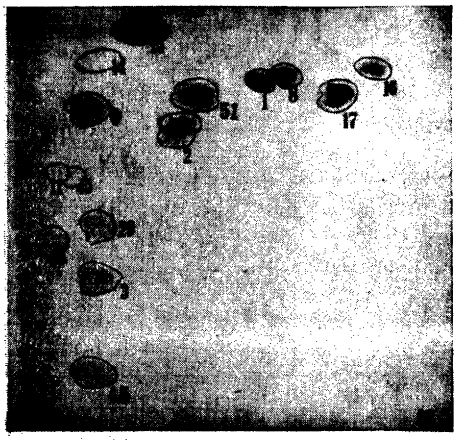


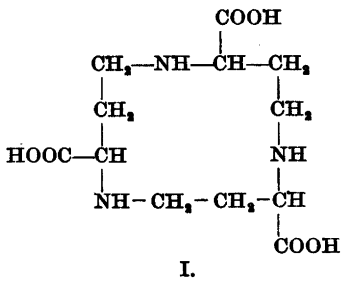
Fig. 3. Treatment of the free amino acid fraction of P. officinale with 4 N NaOH.

using the method of Wendt [2] a molecular weight approximately = 303 was found (Calc. for $(C_4H_7O_3N)_3$: 303.32).

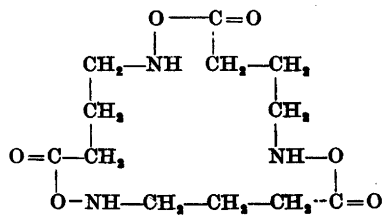
Substance II was prepared and purified in the following way. 926 mg of I were hydrolysed with 6 *N* HCl in a sealed tube for 20.5 hours at 95° C. The reaction products were fractionated on a Dowex 50 column (2 × 42 cm) and the fractions containing mainly II were again hydrolysed to remove traces of I and then deaminated to remove small amounts of homoserine and its lactone. After purification with Amberlite IR-120 some conc. HCl was added and the solution was evaporated to a small volume on a water bath. On precipitation with absolute ethanol 27.8 mg pure hydrochloride of II was obtained. (Found: C 34.23; H 5.84; N 9.93; equiv. wt. 136.0. Calc. for $C_4H_7O_3N$. HCl: C 34.92; H 5.86; N 10.18; equiv. wt. 137.6). Accordingly, II had the same empirical formula as I.

When treated with a saturated solution of $Ba(OH)_2$ II can be changed to I, which then is decomposed to homoserine. Substance I thus gives only homoserine on treatment with alkalies (Fig. 3). It is therefore probable that II, which is formed by acid hydrolysis of I as a byproduct besides the main hydrolysis product homoserine, is some rearrangement product of I. Hence the relationships between I, II and homoserine and its lactone can be shown as depicted below.

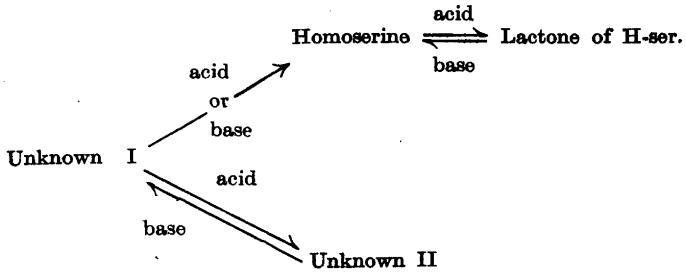
Unknown I has all its carboxyl groups free, but containes no primary or tertiary amino groups. With ninhydrin it liberates the theoretical amount of CO_2 and 55 % of total N as ammonia. It gives a strongly positive reaction for the secondary amino group with sodium nitroprusside and acetaldehyde. Ninhydrin liberates neither



I.



II.



CO_2 nor NH_3 from II, the latter being ninhydrin-positive only on paper, which may be due to some kind of decomposition. However, substance II gives no positive reaction for the secondary amino group as does I. Both I and II are neutral on paper electrophoresis.

The study of these compounds is continued and the experiments and results will be published in detail elsewere. There is, however, now evidence for the structures proposed for I and II.

The following R_F-values were found:

	Phenol-H_2O-NH_3	BuOH-AcOH-H_2O
Lactone of homoserine	0.95	0.35
Unknown I	0.82	0.24
Unknown II	0.66	0.33
Alanine	0.62	0.27
Homoserine	0.57	0.23

When investigating other specimens of the family *Liliaceae*, substance I could be found only in *Convallaria majalis*, *Polygonatum giganteum*, *P. multiflorum*, *Smilacina stellata* and *Majanthemum bifolium*, but not *e. g.* in *Paris quadrifolia*. From a taxonomical point of view this result is very interesting.

We wish to express our deeply felt gratitude to Mr. Andreas Rosenberg, Uppsala for taking the infrared spectra.

1. Giri, K. V. and Nagabhushanan, A. *Naturwiss.* **39** (1952) 548.
2. Wendt, G. *Ber.* **75** (1942) 425.

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New Aminodicarboxylic Acids and Corresponding α-Keto Acids in *Phyllitis scolopendrium*

ARTTURI I. VIRTANEN and ANN-MARIE BERG

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

In a paper by Virtanen and Alfthan [1] the appearance of two new α-keto acids and the corresponding α-amino acids in a fern (*Phyllitis scolopendrium*) was reported. These amino acids formed the spots V and P on a two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3). Both acids were found to be acidic. We have isolated these amino acids and established their chemical nature. At

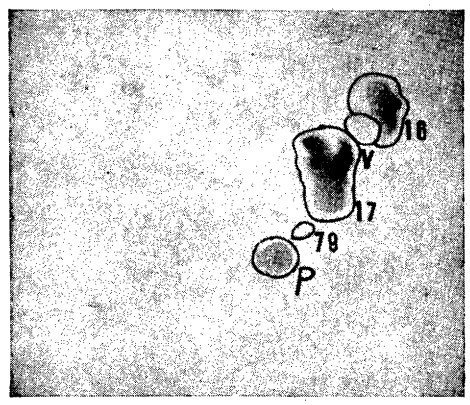

Fig. 1. Two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3) of acidic amino acids in Phyllitis scolopendrium. 16 = asp. V = unknown amino acid, 17 = glu, 79 = γ-methyleneglutamic acid, P = unknown amino acid.

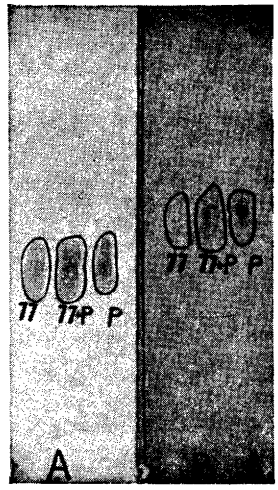

Fig. 2. One-dimensional paper chromatograms of P, synth. γ-methylglutamic acid (77), and a mixture of both. A: butanol-acetic acid, B: phenol-NH_3.

CO_2 nor NH_3 from II, the latter being ninhydrin-positive only on paper, which may be due to some kind of decomposition. However, substance II gives no positive reaction for the secondary amino group as does I. Both I and II are neutral on paper electrophoresis.

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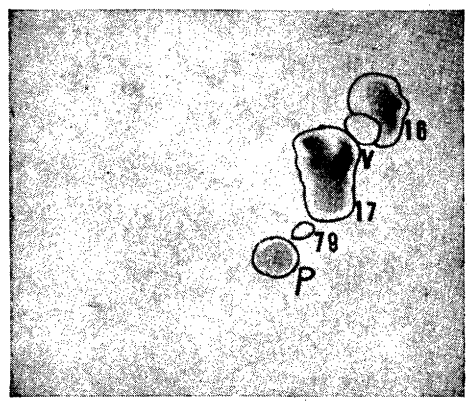

Fig. 1. Two-dimensional paper chromatogram (butanol-acetic acid and phenol-NH_3) of acidic amino acids in Phyllitis scolopendrium. 16 = asp. V = unknown amino acid, 17 = glu, 79 = γ-methyleneglutamic acid, P = unknown amino acid.

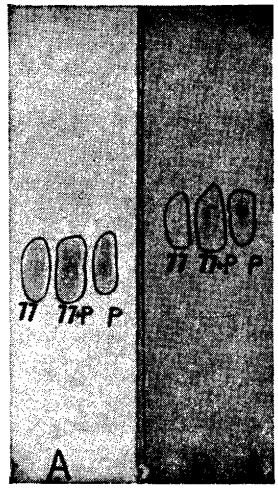

Fig. 2. One-dimensional paper chromatograms of P, synth. γ-methylglutamic acid (77), and a mixture of both. A: butanol-acetic acid, B: phenol-NH_3.

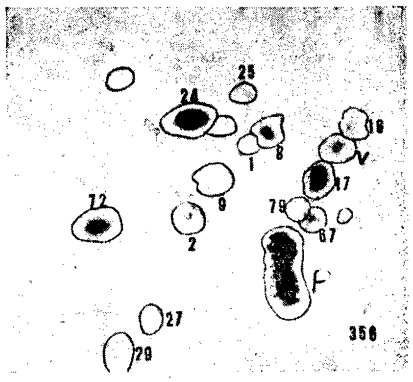

Fig. 3. Two-dimensional paper chromatogram of free amino acids in Phyllitis. 67 = α-aminoadipic acid (added), 79 = methyleneglutamic acid, P = unknown amino acid.

the same time the structure of the corresponding keto acids has been established.

The amino acids were isolated using our earlier method. A 70 % ethanol extract of the plants was run through an Amberlite IR-120 column, and the amino acids remaining in the column were eluted with 1 *N* ammonia. Acidic amino acids were separated from the neutral and basic ones in an Amberlite IR-45 column. The acidic amino acids were eluted from the column with 0.4 *N* hydrochloric acid. The solution was evaporated *in vacuo* to a very small volume. This solution contained aspartic acid, the amino acid V, glutamic acid, γ-methyleneglutamic acid, and the amino acid P (Fig. 1). The acids were separated from each other on the paper chromatogram using butanol-acetic acid as solvent. The separation of P from other amino acids was relatively easy, but the separation of V from aspartic acid and glutamic acid proved to be difficult.

The amino acid P travels with the same velocity as γ-methylglutamic acid (synthetic preparation) when either butanol-acetic acid or phenol-NH_3 are used as solvents (Fig. 2). Analysis of the amino acid P: C 44.88; H 6.87. Calc. for $C_6H_{11}O_4N$: C 44.72; H 6.83; N 8.69. The substance did not contain any OH group. α-Aminoadipic acid travels both with butanol-acetic acid and phenol-NH_3 more slowly than its isomer γ-methylglutamic acid or P (Fig. 3). On the basis of both chromatographic and elementary analysis the amino acid P is γ-methylglutamic acid, $HOOC-CH(CH_3)-CH_2-CHNH_2-COOH$.

When still working on the purification and structure of the amino acid V, we were informed by Dr. F. C. Steward that from the *Adiantum* fern he had isolated a small amount of an amino acid which proved to be identical with synthetic γ-methyl-γ-hydroxyglutamic acid. A small amount of this synthetic acid which Dr. Steward sent in his letter was, according to paper chromatographic analysis, found to be identical also with our amino acid. For the present we have not investigated our acid V more closely chemically. As it is probable that hydroxy-α-aminoadipic acid should travel with the solvents used with about the same velocity as its isomer γ-methyl-γ-hydroxyglutamic acid a later control of the amino acid V is still needed.

On the basis of the results related above the α-keto acids which Virtanen and Alfthan reduced to the corresponding amino acids seem to have the following structure: γ-methyl-α-ketoglutaric acid, $HOOC-CH(CH_3)-CH_2-CO-COOH$, and γ-methyl-γ-hydroxy-α-ketoglutaric acid, $HOOC-C(CH_3)(OH)-CH_2-CO-COOH$.

Correction: In the paper by Virtanen and Alfthan [1], p. 189, "acidic OH-containing amino acid P and — — — of another amino acid V" should according to the manuscript be: acidic amino acid P and ——— of another OH-containing amino acid V.

We are indebted to Dr. L. Fowden, London, for a sample of synthetic γ-methylglutamic acid, and to Dr. F. C. Steward, New York, for γ-methyl-γ-hydroxyglutamic acid.

1. Virtanen, A. I. and Alfthan, M. *Acta Chem. Scand.* **9** (1955) 188.

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The Anomalous Reactivity of *o*-Methoxyphenylmagnesium Bromides

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In the reactions between carbon dioxide and the Grignard reagents prepared from *o*-methoxybenzyl chloride, *o*-bromodimethylaminobenzene, and *o*-bromomethylthiobenzene, both the corresponding acids and the corresponding ketones are formed. The Grignard reagents prepared from β-(*o*-methoxyphenyl)ethyl chloride and γ-methoxypropyl bromide yield only the corresponding acids under the same conditions. The anomalous reactivity leading to ketone formation is associated with the formation of strained planar rings through a complex bond between the magnesium atom and the nitrogen, oxygen, or sulphur atoms. Such rings are not likely to be formed by the molecules of the above-mentioned two Grignard reagents which react normally. Steric hindrances of different kinds seem to be involved in other previously described cases in which the anomalous reactivity is cancelled.

Some time ago, the author showed that in the reaction between *o*-methoxyphenylmagnesium bromide and carbon dioxide 2,2′-dimethoxybenzophenone is formed in addition to *o*-methoxybenzoic acid, and that a similar ketone formation occurs when a methyl or methoxy substituted *o*-methoxyphenylmagnesium bromide reacts with carbon dioxide unless the second substituent is in the ortho position relative to the original methoxy group or to the magnesium atom [1,2]. It was concluded that the ketone formation is an exceptional reaction of the Grignard reagent.

When the investigations were extended to *o*-methoxybenzylmagnesium chloride and β-(*o*-methoxyphenyl)ethylmagnesium chloride, it was found that the former gave the corresponding ketone (isolated only as its 2,4-dinitrophenylhydrazone) and the corresponding acid, whereas the latter gave β-(*o*-methoxyphenyl)propionic acid as the only product of the reaction. These reaction products seem to indicate that the exceptional reactivity is connected with ring formation in such a way that one of the two "etherate" ether molecules of the Grignard reagent has been displaced by the methoxy group, *i.e.* *o*-methoxybenzylmagnesium chloride and *o*-methoxyphenylmagnesium bromide have the constitutions shown by formulae I and II.

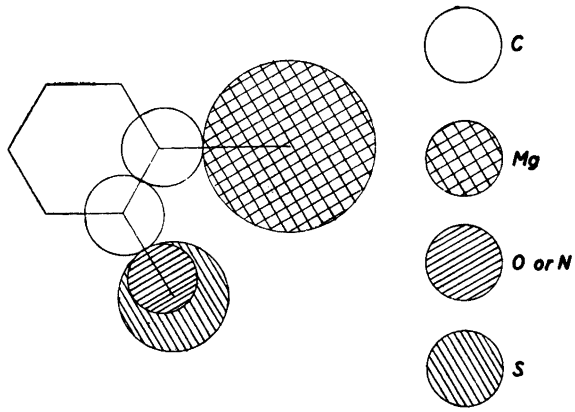
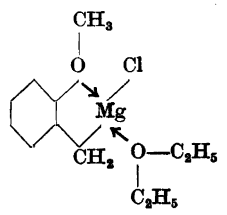
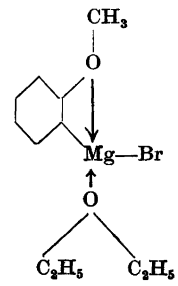


Fig. 1. The theoretical positions of the atoms in o-*methoxyphenylmagnesium bromide,* o-*dimethylaminophenylmagnesium bromide, and* o-*methylthiophenylmagnesium bromide as based on the assumption that the bond lengths and the valence angles have the usual values and that attractive forces do not exist between the Mg atom on one side and the O, N, and S atoms on the other. The radii of the circles are proportional to the bond lengths of the atoms. Atoms not participating in the reaction have been omitted.*

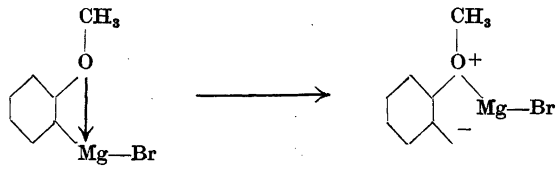


I



II

That these ring formations are possible with all atoms in one plane is shown in Figs. 1 and 2. From these figures it will also be seen that the rings must be under strain, at least with respect to the valence angles. In the case of β-(*o*-methoxyphenyl)-ethylmagnesium chloride the ring would contain too many atoms. The intramolecular strained ring may be expected to favour the polarization of the carbon-to-magnesium bond and thus to increase the reactivity of the compound.



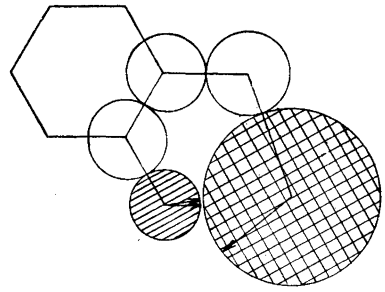


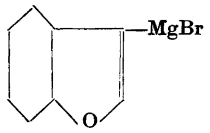
Fig. 2. The theoretical positions of the atoms in o-methoxybenzylmagnesium chloride when the distance between Mg and O atoms is a minimum as based on the assumptions stated in Fig. 1. The arrows indicate possible directions of the complex bonds.

If this hypothesis is correct, one may expect that β-methoxyethylmagnesium bromide and other non-aromatic Grignard reagents with an alkoxy group in the β position relative to the magnesium atom would possess an exceptional reactivity of the type in question, provided that the magnesium atom and the ether group are in the "*cis*" position relative each other. Unfortunately such Grignard reagents cannot be prepared since any attempt in this direction leads to the formation of unsaturated compounds [3-5]. Mann and Steward [6] have recently discussed reactions of this type and proposed the following mechanism for β-alkoxyethylmagnesium bromide:

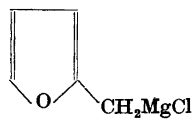
$$RO{-}CH_2{-}CH_2{-}Br + Mg \rightarrow RO{-}CH_2{-}CH_2{-}MgBr \rightarrow CH_2{=}CH_2 + RO{-}MgBr$$

The hypothesis proposed above for the exceptional reactivity of *o*-methoxyphenylmagnesium bromide and related compounds requires that β ether Grignard reagents with the oxygen atom and the magnesium atom in the "*trans*" position relative to each other should give acids but no ketones in the reaction with carbon dioxide. Compounds of this kind are not generally stable but decompose according to the mechanism of Mann and Steward. The only Grignard reagent of this type that appears to have been synthesized (although in poor yield) is 3-bromobenzofurylmagnesium bromide (III). Reichstein and Baud [7] isolated from the products of the reaction between this Grignard reagent and carbon dioxide chiefly *o*-hydroxyphenylacetylene (formed according to the mechanism of Mann and Steward) and a small amount of 3-benzofuroic acid. No ketone seems to have been formed, which supports the above hypothesis.

The fact that aromatic Grignard reagents do not decompose according to the mechanism of Mann and Steward is, of course, due to the stabilization of



III



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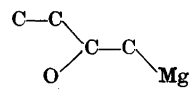

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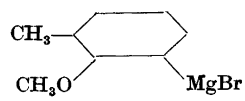

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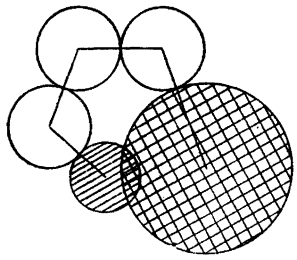
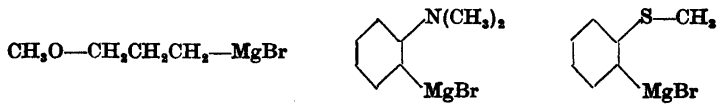


Fig. 3. The structure of an alkoxyethylmagnesium halide computed as for the Grignard reagents in Fig. 1 and 2.

the bonds in the aromatic nucleus. A similar stabilization might be expected in furfurylmagnesium chloride (IV). This compound is not, however, well adapted for an investigation of the present type, because a Wurtz' type of reaction seems to predominate in the reaction between magnesium and furfuryl chloride[8]. Further, even if the corresponding ketone would not be formed in the reaction between this Grignard reagent and carbon dioxide, this could be explained by noting that the compound in question has a constitution that in certain respects resembles that of 3-methyl-2-methoxyphenylmagnesium bromide (VI), which does not give the corresponding ketone in the reaction with carbon dioxide. An examination of the constitutions of the two substances (IV and VI) and formula V, which gives the part that is common to both, shows that the carbon atom in position 4 of the furyl derivative corresponds to the carbon atom in the methyl group of the benzene derivative. Introduction of this latter methyl group cancelled the anomalous reactivity of *o*-methoxyphenylmagnesium bromide, and one might expect that the carbon atom in position 4 would also cancel a potential anomalous reactivity of the furan derivative.

A chelate formation of the type in question is not likely in γ-methoxypropylmagnesium bromide (VII; *cf.* Fig. 3). It may thus be expected that this Grignard reagent will give only the corresponding acid in the reaction with carbon dioxide. This prediction was experimentally confirmed.

Finally, if the hypothesis proposed above is correct the exceptional reactivity of the type in question should be possible also in cases in which the methoxy



VII VIII IX

group is replaced by other groups with unshared electrons on the central atom, which are able to form a complex bond with the magnesium atom similar to that formed between magnesium and the oxygen in the methoxy group. Compounds of this type, *o*-dimethylaminophenylmagnesium bromide (VIII) and *o*-methylthiophenylmagnesium bromide (IX) were studied. Both gave the corresponding acids and the corresponding ketones in accordance with the hypothesis.

On the basis of the above it seems justified to suppose that the ring formation is the explanation for the exceptional reactivity of *o*-methoxyphenylmagnesium bromide and other Grignard reagents with unshared electron pairs which are able to form a bond with magnesium, which leads to the formation of a strained planar ring.

As mentioned above, *o*-methoxyphenylmagnesium bromides do not give the corresponding ketones in the reaction with carbon dioxide if there is a methyl or methoxy group in the *ortho* position relative to the methoxy group or to the magnesium atom. A molecular model study shows that in the first case the methoxy group is not able to rotate freely. In the second case, a steric hindrance similar to that existing in mesityl compounds is probable.

EXPERIMENTAL

o-Methoxybenzyl chloride was obtained from *o*-methoxybenzyl alcohol which was prepared from ethyl *o*-methoxybenzoate.

Lithium aluminium hydride (7.5 g; technical grade) and dry ether (500 ml) were placed in a flask (1 l), which was equipped with a mercury-sealed stirrer, a reflux condenser, and a dropping funnel. The stirrer was started and after five hours a solution of ethyl *o*-methoxybenzoate [9] (57.8 g) in dry ether (200 ml) was added through the dropping funnel at such a rate that the ether refluxed gently. When the addition was complete, the reaction mixture was warmed on a water bath for three quarters of an hour. The excess of lithium aluminium hydride was destroyed by adding ethyl acetate and the reaction products were hydrolyzed by adding water. The reaction mixture was then poured into a solution of concentrated hydrochloric acid (100 ml) in water (1 l) in order to dissolve the precipitated aluminium hydroxide. The ether phase was removed and the aqueous phase was extracted with a second portion of ether. The ether solutions were combined and dried with anhydrous sodium sulphate. When the solvent had been evaporated, *o*-methoxybenzyl alcohol, b. p. 126—127°/15 mm, was obtained by distillation. The yield was 77.5 % (34.5 g).

A small amount of this alcohol was converted into the corresponding *p*-nitrobenzoate. The melting point of the ester was 81—82°, as reported by Mozingo and Folkers [10].

o-Methoxybenzyl alcohol (33.2 g) was dissolved in chloroform (50 ml) and added to a flask (250 ml) equipped with a reflux condenser and a dropping funnel, through which a solution of thionyl chloride (30 g) in chloroform (50 ml) was gradually added. When addition was complete, the reaction mixture was heated to boiling until evolution of gas ceased. The solvent was distilled off under normal pressure and the residue under reduced pressure. The yield of *o*-methoxybenzyl chloride, b. p. 108—109°/15 mm, was 35.5 % (13.7 g).

β-(o-Methoxyphenyl)ethyl chloride was prepared according to Hill, Short, and Stromberg [11] from β-(*o*-methoxyphenyl)ethyl alcohol, which was obtained from *o*-methoxyphenylmagnesium bromide and ethylene oxide according to Bogert and Hamann [12].

A small amount of the alcohol was converted into the corresponding *p*-nitrobenzoate. This latter substance melted at 58—59°. (Found: N 4.77. Calc. for $C_{16}H_{15}O_5N$: N 4.65).

γ-Methoxypropyl bromide. γ-Methoxypropyl alcohol was prepared from trimethylene glycol and methyl iodide according to Smith and Sprung [13]. The alcohol was then converted into the chloride according to Pummerer and Schönamsgruber [14].

o-Bromodimethylaniline was prepared according to Gilman and Banner [15] from *o*-bromoaniline in 78.2 % yield. *o*-Bromoaniline was obtained by the reduction of *o*-bromonitrobenzene with stannous chloride in the way previously described for 5-amino-3-bromoveratrole [3]. After distillation under reduced pressure, the yield of *o*-bromoaniline, b. p. 98°/11 mm, was 78.2 %.

o-Bromomethylthiobenzene. o-Bromothiophenol (48 g), prepared according to Schwarzenbach and Egli [16] from *o*-bromoaniline, was dissolved in an aqueous solution (100 ml) of sodium hydroxide (10.5 g) and the mixture was shaken with dimethyl sulphate (32 g). When the mixture had cooled, dimethyl sulphate (16 g) and a dilute solution of sodium

hydroxide (5.3 g) in water (50 ml) were added and shaking was continued until the mixture had cooled again. A solution of sodium hydroxide (12 g) in water (50 ml) was now added and the mixture was heated for half an hour on the boiling water bath. After cooling, the oil was taken up into ether and the water solution was extracted once more with ether. The combined ether solutions were dried with anhydrous sodium sulphate and gave after distillation under reduced pressure a 88.5 % yield (48 g) of pure *o*-bromomethylthiobenzene, b. p. 145—146°/27 mm.

The reactions between Grignard reagents and carbon dioxide were examined as has been described previously [1]. The results are given below.

o-Methoxybenzylmagnesium chloride (prepared from 5 g of the corresponding halide) gave 0.91 g of *o*-methoxyphenylacetic acid, m. p. 124—125°, and 2.19 g of neutral substances. When the latter were treated with alcohol, 0.80 g of 2,2′-dimethoxydibenzyl (m. p. after recrystallization from alcohol 85—86°) were obtained. The filtrate was evaporated to dryness and the residue (1.20 g) treated with 2,4-dinitrophenylhydrazine. From the mixture the hydrazone (1.02 g) of 1,3-di-(*o*-methoxyphenyl)acetone was isolated. After recrystallization from alcohol, the substance melted at 157.5—158°. (Found: N 12.32. Calc. for $C_{23}H_{22}O_6N_4$: N 12.44.)

A small amount of the *o*-methoxyphenylacetic acid was converted into its *p*-nitrobenzyl ester. This substance melted at 64.5—65°. (Found: N 4.51. Calc. for $C_{16}H_{15}O_5N$: N 4.65.)

β-(*o*-Methoxyphenyl)ethylmagnesium chloride (prepared from 5 g of the corresponding halide) gave 1.55 g of *β*-(*o*-methoxyphenyl)-propionic acid, m.p. 88—89°. The *p*-nitrobenzyl ester of the latter melted at 45—46°. (Found: N 4.41. Calc. for $C_{17}H_{17}ON$: N 4.44.) No ketone could be isolated.

γ-Methoxypropylmagnesium bromide (prepared from 5 g of the corresponding halide) gave 0.89 g of *γ*-methoxybutyric acid. Its *p*-phenylphenacyl ester melted at 79—81°, in agreement with the value found by Owen and Sultanbawa [17]. No ketone was detected.

o-Dimethylaminophenylmagnesium bromide (prepared from 5 g of the corresponding halide) was treated with carbon dioxide in the usual manner. The reaction mixture was treated with dilute hydrochloric acid and the ether was separated (residue of neutral substances 0.04 g). The aqueous layer was made alkaline with aqueous ammonia, ether was added, and the mixture was filtered. The layers in the filtrate were separated. When the solvent had been evaporated from the ether phase, 1.94 g of 2,2′-di-(dimethylamino) benzophenone were isolated. After recrystallization from ligroin, it melted at 120—121°. With picric acid it formed an addition compound that melted at 150—151°. The aqueous solution was acidified with acetic acid and extracted with ether in an extractor for several hours. When the residue from the ether solution was treated with ether 0.70 g of dimethylanthranilic acid was obtained.

o-Methylthiophenylmagnesium bromide (prepared from 5 g of the corresponding halide gave 1.10 g of *o*-methylthiobenzoic acid, m.p. 168—169°, and 1.43 g of neutral substances. When the latter were treated with benzene 2,2′-dimethylthiobenzophenone separated. After recrystallization from ethyl alcohol, the latter melted at 105—106°. (Found: S 23.55. Calc. for $C_{15}H_{14}OS_2$: S 23.37.) The same substance was obtained by hydrolyzing the reaction product from the reaction between *o*-methylthiophenylmagnesium bromide and *o*-methylthiobenzonitrile but not by decomposing *o*-methylthiophenylmagnesium bromide with water.

A small amount of the *o*-methylthiobenzoic acid was converted into its *p*-nitrobenzyl ester. The melting point of this ester was 122—123°. (Found: N 4.71. Calc. for $C_{15}H_{13}O_4NS$: N 4.62.)

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A Microbiological Assay of Vitamin B_1

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A microbiological assay for thiamine, using the yeast *Kloeckera brevis* is described. The method is simple and specific. The sensitivity is high enough to allow the determination of 0.001 μg of thiamine per ml of extract with a standard deviation of less than 10 %.

Although a large number of methods for the determination of thiamine (vit. B_1) has been described, each has certain limitations.

Modifications of Jansens [1] fluorometric thiochrome method seems to be preferred in most laboratories, although this method is neither as specific nor as sensitive as the microbiological methods.

The only microbiological assay which seems to have been generally used is modifications [2] of the *Lactobacillus fermentum* method, originally described by Sarett and Cheldelin [3]. *L. fermentum*, however, is very exacting and some laboratories including our own have been unable to obtain consistent satisfactory results with this organism.

We have therefore investigated a number of thiamine requiring microorganisms in search of a less exacting strain which could be used both for the routine analysis of thiamine in natural products and in an investigation of the effect of various factors on the destruction of this vitamin. Of the organisms tested *Kloeckera brevis* gave the most promising results. Burkholder [4] has shown that this yeast strain requires six members of the B-vitamin group for optimal growth and he has recommended this organism for the assay of inositol. Our preliminary tests, however, showed that none of the media described for *Kloeckera brevis* allow maximum growth in a thiamine assay. The main problem therefore was to devise assay conditions under which thiamine is the only limiting factor for growth.

METHOD

a. *Organism.* *Kloeckera brevis* B 768, obtained from *The National Collection of Industrial Bacteria*, Teddington, Middlesex, U.K.

b. *Agar medium.* Dissolve 20 g of malt extract, to which no preservative has been added, in 200 ml of water. Adjust the pH to 5.0 and dissolve 5.0 g of agar in the mixture at about 100° C. Dispense the medium in 5 ml quantities to test tubes. Plug

the tubes with cotton, autoclave at 120° C for 15 min., slope, and store in a refrigerator. After transfer incubate the cultures at 30° C for 16—24 h and keep them in a refrigerator until the next transfer. Transfer the stock cultures at monthly intervals.

c. *Inoculum.* Remove about 2 mg of wet yeast cells with a bacteriological loop from a 16—24 h old culture and suspend in 10 ml of sterile tap water. Dilute 1 ml of this suspension with 10 ml of sterile tap water. Use 0.05 ml of this diluted cellsuspension for the inoculation of each tube.

d. *Basal medium.*

0.5	g	casamino acids (Difco)
4.0	g	glucose
10	ml	salt E
5	»	trisodium citrate · 5 H_2O solution, 15 % w/v
2	»	L-asparagine solution, 2 % w/v
5	»	yeast extract solution
1	»	vitamin solution

Mix the solutions, add 50 ml of water and dissolve the glucose and the casamino acids in the mixture. Adjust the pH to 4.7 with 1 *N* HCl and add water to make 100 ml.

Prepare the solutions as follows:

Salt E. Dissolve 22 g KH_2PO_4, 17 g KCl, 10 g $(NH_4)_2SO_4$, 4 g $CaCl_2$, 4 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $FeCl_3$, 0.1 g $MnSO_4 \cdot 4\ H_2O$ and 10 ml conc. HCl in water to make 1 000 ml.

Vitamin solution. Dissolve 100 mg *meso*-inositol, 20 mg choline chloride, 50 μg (+) biotin and 5 mg each of nicotinic acid; pyridoxine chloride and calcium pantothenate in water to make 50 ml. Store under a preservative in a refrigerator. Prepare a fresh solution every month.

Yeast extract solution. Dissolve 5 g of yeast extract (Difco) and 0.25 g of $Na_2S_2O_3 \cdot 7\ H_2O$ in 50 ml of water, adjust the pH to 5.4 and autoclave the mixture at 120° C for 30 min. Cool the solution and add an exact amount of 2 % H_2O_2 solution (about 6 ml) to oxidize excess sulfite. A freshly mixed solution of equal parts of 5 % KI solution, 1 % starch solution and 50 % w/v sulfuric acid solution is used as an external indicator. Dilute the yeast extract solution with water to make 100 ml. Store under a preservative in a refrigerator. Prepare a fresh solution every month.

Standard thiamine solution, 0.0025 μg per ml. Dry thiamine dichloride (M 337.3) over H_2SO_4 in vacuum to constant weight.

Stock solution: Dissolve 100 mg of the dried vitamin in 50 ml of 0.1 *N* HCl and add water to make 100 ml. Store in a refrigerator. Prepare a fresh solution every month. The standard solution is prepared fresh every day by diluting 250 μl (constriction pipette) of the stock solution with water to make 100 ml.

e. *Assay procedure.* The assay is carried out in lipless uniform test tubes (100 × 16 mm. i.d.). To each series the standard thiamine solution (2.5 mμg per ml) is added in the following amounts: 0, 0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml, each with an error of not more than 2 %. Each level is set up in triplicate. The sample to be assayed is similarly added to a series of tubes in the following amounts: 0.3, 0.5, and 0.8 ml in duplicate. Water is added to the tubes to make a total volume of 1 ml, and 0.95 ml of the basal medium is added. The tubes are shaken to mix the content, covered with a cloth, steamed at 100° C for 10 min., cooled and inoculated. In each set of standard tubes one of the tubes containing 0 ml of standard solution is left uninoculated. These tubes are used as blanks in the turbidimetric determination of growth. The tubes are incubated at 30° ± 0.5° C for 18—24 h in a *shaking machine* to guarantee sufficient aeration and uniform growth.

f. *Determination of response.* The tubes are shaken and the cultures transferred to a microcuvette. The turbidity is read at λ = *ca.* 650 mμ in a suitable photometer (*e.g.* Lumetron 402 E).

g. *Calculation of results.* A standard dose-response curve is prepared by plotting the average of the turbidity values found at each level of the thiamine standard against the amount of thiamine present (*Cf.* Table 1 and Fig. 1). The vitamin content of a sample is determined by interpolating the response to the known amounts of the test solution onto this standard curve. The thiamine content per ml of the test solution is now calculated for each duplicate sets of tubes, and the thiamine content of the sample is calculated from the average of the values.

h. *Preparation of samples for assay.* Enough of the material to give a representative sample is suspended in at least 25 ml of 0.1 *N* sulfuric acid per g dry matter. The mixture is steamed for 30 min. at 100° C and the pH adjusted to 4.7 with 2.5 *M* sodium acetate. 100 mg of mylase P (Wallerstein Lab., N.Y.) or another suitable enzyme preparation is added per g of dry matter. The mixture is covered with a preservative and incubated at 37° C for 18—24 h. The digest is then steamed for 10 min. at 100° C and diluted with water to a suitable volume. After filtering through filterpaper the clear filtrate is diluted to contain 1—2 mμg of thiamine per ml. The content of thiamine in each batch of enzyme preparation must be determined and if significant taken into account.

i. *Reproducibility of the assay.* The standard deviation of repeated independent assays of several crude materials has been 5—8 %.

RESULTS

a. *Effect of yeast extract on growth.* For some time we used the basal medium described without the addition of the sulfite treated yeast extract after having convinced ourselves that neither the addition of other substances known to be growth factors for microorganisms (vitamins, purines, pyrimidines, nucleotides, deoxynucleotides, salts, amino acids *etc.*) nor the addition of alkalitreated peptone, malt extract and yeast extract enhanced the growth. Sometimes, however, assays of crude material showed positive "drift" and occasionally results seem to be too high. As shown by Williams [5] sulfite under rather mild conditions destroys the thiamine activity by splitting the thiamine molecule. We therefore tested the growth effect of some sulfitetreated natural products and found that yeast extract and malt extract in which the thiamine was destroyed by sulfite did actually enhance the growth (Fig. 1). After the addition of an optimal amount of sulfitetreated yeast extract thiamine seems to be the only limiting factor for growth as no "drift" occurred in the assay of natural products as exemplified in Table 1.

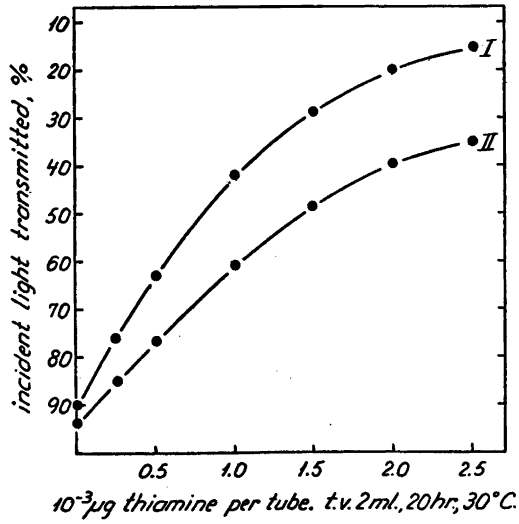

Fig. 1. Growth curves for Kloeckera brevis. I Medium as described. II Medium as described without yeast extract.

Table 1. Determination of thiamine in beef. 5 g of minced beef extracted as described. Final solution ~ 1.25 mg beef per ml. Total volume: 2 ml per tube.

Standard					Beef extract				
Thiamine	Turbidity				Beef	Turbidity			Thiamine
mμg per tube	ser. 1	ser. 2	ser. 3	mean	mg per tube	ser. 1	ser. 2	mean	mμg/mg beef
0	90	89	90	90	0.125	82	80	81	1.12
0.25	75	77	76	76	0.25	72	71	72	1.20
0.5	63	64	62	63	0.50	63	61	62	1.02
1.0	42	(90)	42	42	0.75	44	44	44	1.22
1.5	29	30	28	29	1.00	39	37	38	1.12
2.0	21	19	20	20	1.25	32	(12)	31	1.05
2.5	(21)	14	15	15					
							mean:		*1.12*

b. *Response to cocarboxylase and to the pyrimidine and thiazole moities of thiamine.* On a molar basis cocarboxylase (thiaminepyrophosphate) has about 30 % less growth promoting activity as thiamine. After treatment with mylase, as described for the preparation of samples for assay, the response to cocarboxylase is the same as to thiamine.

For several microorganisms the growth effect of thiamine can be substituted by either both or one of the two main fission products of thiamine namely, 2-methyl-4-amino-5-aminomethyl-pyrimidinedichloride and 4-methyl-5-(β-hydroxyethyl)thiazole. For mammals these two naturally occurring substances are without vitamin activity. The same holds true for *Kloeckera brevis*. Even after incubation for 72 h neither of the two substances nor a mixture of both can substitute thiamine for growth. At least up to concentrations 10 times the maximum concentration of thiamine used in the assay, they have no inhibitory effect either.

Table 2. Results of analysis of some products for their thiamine content by two methods.

Product	Thiamine μg/g	
	Kloeckera brevis method	Thiochrome method
Wheat flour	1.85	1.65
» » vitaminized	5.60	5.25
Rye bread	2.20	2.30
Fodder additive A	58.5	58.0
Liquor B-vitaminorum	48.0	45.5
Fodder yeast 1.	1.90	1.75
» » 2.	2.70	2.45
» » 3.	11.5	12.0
Fodder additive B	110	115

c. *Comparison of results obtained by the Kloeckera brevis method and the thiochrome method.*
As seen from Table 2 the results of the two methods agree resonably well.

The authors wish to express their thanks to Dr. W. Hjarde, Statens Vitaminlaboratorium, Copenhagen, for the fluorometric analysis.

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On Adsorption of Alkaloids

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Experiments on the behaviour of alkaloidal salts on columns of the artificial permutit Decalso F have given the following results:

1. In alkaloidal and similar salts in aqueous solution the cations are exchanged for potassium, and monovalent anions go straight through. Multivalent anions are retained stronger.
2. Alkaloidal salts soluble in organic solvents, *e.g.* chloroform, are most often retained as a whole.
3. Subsequent elution with water releases the anion.
4. Free alkaloidal bases dissolved in *e.g.* chloroform are adsorbed to the Decalso.

Decalso and other permutoid compounds have frequently been used for water softening by ion exchange, calcium and magnesium being exchanged for sodium or potassium. It seems likely that alkaloidal salts in aqueous solution will behave in a similar way. In recent papers [1,2] it has been established that the cations of such salts are adsorbed by the Decalso and in the present paper evidence is given that both ions follow the general rules valid for ion exchange processes.

Hyoscine hydrobromide, atropine sulphate, and codeine phosphate were adsorbed on Decalso columns from aqueous solutions [1,2]. The recoveries of the anions are shown in Table 1.

The bromide is very easily eluted, the sulphate requires much more water and some losses appear. The phosphate is most strongly adsorbed and could only be recovered in 83 % yield with twenty times as much water as for the bromide. Subsequent elution with 0.2 *N* hydrochloric acid did only add 7 % to the yield of phosphate in the eluate, and 0.2 *N* acetic acid nothing. For alkaloidal cations these acids are very effective eluants [1,2]. These findings concord with the general ion exchange experience.

As cation only potassium could be detected in the water effluent from columns charged with hyoscine hydrobromide. The recoveries were between 121 and 129 % calculated on the amount equivalent to the alkaloid. The values are difficult to explain but might be due to a slight decomposition of the Decalso lattice. We are, however, sure that the discrepancy is not due to experimental errors.

Previously we have shown [1] that the free bases of atropine and hyoscine extracted from their salts could be adsorbed by Decalso from ethereal solutions. The bases were in those cases liberated with ammonia or sodium carbonate. Now, a similar technique has been applied to a number of other bases. Most bases investigated were quantitatively retained by the Decalso from solutions in chloroform or ethyl ether and could be eluted in excellent yields with acids as before (Table 2). It did not matter whether the organic solvents were dry or saturated with water or whether the Decalso column was prepared with the exclusion of water or not. If, however, the organic solvent contained too much ammonia the alkaloid leaked through: the more ammonia the more leakage.

Some organic salts, *e.g.* some alkaloidal salts of inorganic acids, are soluble in organic solvents such as chloroform and ether. Such solutions were applied to Decalso columns and the effluents were tested for cations and anions. Most of the salts investigated were retained by the Decalso, *i.e.* not only the cation but also the anion. This is a most interesting fact which indicates that the process involved is hardly a common ion exchange.

If the column charged with the salt is washed with water the anion is eluted as before. The cation sticks to the Decalso but can as usual be eluted with acids.

An inspection of the Tables 2 and 3 and the previous paper [1] reveals that some bases and some salts do not behave in the same way as the majority of the compounds tested. Bases as atropine [1], hyoscine [1], codeine, quinine, strychnine, and even 2-aminopyridine (pK_b in water ranging from 3.3 to 6.9) were adsorbed by the Decalso both as free bases and salts from chloroform, ether, or water. Weaker bases, however, as N-diethylaniline, pyridine, and 1-naphthylamine (pK_b in water 7.4—10.0) leaked through, the weaker the more. Thus, there seems to be a certain limit as to the strength of the bases which can be taken up by the Decalso.

EXPERIMENTAL

Table 1. Desorption of anions.

Alkaloidal salt	Elution with water ml	Recovery of anion %
Hyoscine hydrobromide	15	99.1; 98.7
Atropine sulphate	40	95.0; 98.7
Codeine phosphate	50—300	45—83

10—15 mg of the alkaloidal salt was adsorbed on 2.0—2.5 g of Decalso * in a column 1 cm wide [1]. The column was washed with varied amounts of water and the anion determined in the effluents. Blanks were always run.

Bromide. The eluate was evaporated to near dryness after the addition of 0.50 ml of 0.1 *M* sodium bicarbonate solution and titrated with 0.1 *N* silver nitrate according to Volhard in the presence of nitrobenzene.

* Decalso F, obtained from the Permutit Company, Gunnersbury Av., London, W. 4. through Rudolph Grave, Stockholm.

Sulphate. The eluate was acidified with 1.0 ml of 2 *M* hydrochloric acid and the sulphate precipitated with 5.00 ml of 0.2001 % barium chloride solution. The mixture was evaporated to dryness on the waterbath, 5.00 ml of 0.1316 % potassium dichromate solution and 3.0 ml of 5 % sodium acetate solution were added. After evaporation to dryness on the waterbath the residue was stirred with 25.00 ml of water, centrifuged and 10.00 ml of the supernatant liquid withdrawn and after evaporation to about 2 ml the excess of potassium dichromate was titrated iodometrically with 0.1 *N* sodium thiosulphate.

Phosphate. The phosphate was determined both colourimetrically [4] and acidimetrically [5]. As the values obtained were very irregular and low we tried wet ashing with a mixture of nitric and sulphuric acids and dry ashing with sodium carbonate and potassium chlorate. These attempts did not improve the results on the effluents. When the various techniques were applied on plain codeine phosphate or potassium phosphate however good results were obtained.

Potassium was determined with a Zeiss flame spectrophotometer. Standard curves were made with solutions of potassium bromide. To all solutions lithium chloride was added to a final concentration of 0.1 mg per ml, *i.e.* about 10—20 times the amount of potassium.

During determinations frequent standards were run and in each sample also the sodium content was determined. In no case, however, measurable amounts of sodium were detected.

Preliminary experiments showed that it was impossible to obtain a potassium-free effluent from plain Decalso with water. However, after copious washing the potassium leakage became constant under the conditions of each experiment. Hence, the Decalso (2.50 g) was soaked overnight in water, and then washed in the column with 3—4 50 ml portions of water until the potassium content of the effluent had become constant. About 15.00 mg of hyoscine hydrobromide dissolved in 10 ml of water was adsorbed on the Decalso. The column was washed with water, the effluent being collected in 50.0 ml volumetric flasks. The potassium concentration was high in the first 50 ml eluate, but dropped in the second to the same value as before the adsorption. As checks blank columns were run parallel.

Example:	Uncorrected recovery	39.5	μg K/ml
	Constant leakage	1.5	»
	Corrected value	38.0	»
	Theory	32.45	»

As the values were always 20—30 % high wet ashing with perchloric and nitric acids was tried, but the same results were obtained.

The substance was dissolved in some ml of the solvent in question and the solution passed through the decalso column [1,2]. The column was washed, first with 10—15 ml of the solvent, then with 10—15 ml of water. If an organic solvent was used it was displaced

Table 2. Adsorption of bases on Decalso, 0.5 or 0.6 g.

Alkaloid	pK_b (water)	Amount mg	Solvent	Recovery (%) after elution with 0.2 *N* acetic or hydrochloric acid	
Codeine	6.05	4.8	Ether	100.2	99.2
»		5	Chloroform	99.5	100.3
Quinine	5.9	1.3	Ether	100.3	99.7
»		1.5	Chloroform	99.0	99.5
Strychnine	6.0	1.0	Chloroform	99.5	99.6
2-Aminopyridine	6.9	0.3	Chloroform	100.2	99.7
N-Diethylaniline	7.4	1.0	Chloroform	15—20	
Pyridine	8.9	1.1	Water	Total leakage	
1-Naphthylamine	10.0	1.5	Chloroform	2—17	

Table 3. Adsorption of salts on Decalso.

Alkaloidal salt	pK_b base in water	Amount		Solvent	Recovery (%) after elution with 0.2 *N* acetic or hydrochloric acid		
		Alkaloidal salt mg	Decalso g				
Dimethylammonium chloride	3.3	8	3	Chloroform	97.6	100.5	100.5
Hyoscine hydrobromide	6.2	1.4	0.25	»	97.0	97.5	
Papaverine hydrochloride	8.1	1.3	0.6	»	99.0	99.6	
		1.6	0.6	»	98.8	99.2	
2-Aminopyridine hydrochloride	6.9	0.2	0.5	Water	100.1	101.6	100.6
N-Diethylaniline hydrochloride	7.4	1.0	0.5	»	94.4	92.6	
Pyridine hydrochloride	8.9	1.1	0.5	»	Total leakage		
1-Naphthylamine hydrochloride	10.0	0.8	0.5	Ether	72.8	73.7	
		0.7	0.5	Water	Total leakage		
Tetraphenylboron compound of methylhyoscine [3]	strong	30	2.5	Acetone	100.9	100.6	

by some alcohol prior to the water. If the solvent was water the first 10—15 ml of effluent was examined separately. When chloroform or ether were used the organic phase and the alcoholic-aqueous phases were separately examined. The organic effluents were evaporated together with some water and the aqueous residue was investigated.

The cations were eluted from the columns with acids [1,2] and determined in the acid solution with the aid of their ultraviolet absorption. Diethylaniline was extracted with 2,2,4-trimethylpentane from the alkalinized effluent and measured in the hydrocarbon. Dimethylamine was determined by a micro-Kjeldahl procedure.

In addition to the quantitative experiments listed in Table 3 qualitative tests were made on chloroform solutions of the following salts:

Hyoscine hydrobromide	pK_b for the base in water	6.2
Papaverine hydrochloride		8.1
Narcotine hydrochloride		7.8
Atropine sulphate		4.4
Dimethylammonium chloride		3.3
Physostigmine salicylate		6.1
Alypine hydrochloride		4.5
Homatropine hydrobromide		~ 4.7

The chloroform effluents were evaporated with some water and the water tested for the anion in question, in each case with a negative result. On the other hand the anions were present in the subsequent aqueous eluates.

In one case, hyoscine hydrobromide, the anion was quantitatively determined in the aqueous eluate as previously described. The recoveries were: 100.6, 99.1, and 99.3 %.

We are indebted to Messrs. Pharmacia Ltd. for permission to publish this paper and to Mr. L. Magnusson who carried out most of the experimental work.

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Countercurrent Fractionation of Cellulose Acetate by Preferential Diffusion into Porous Charcoal

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A multi stage countercurrent apparatus is described with which porous charcoal is used as the stationary phase. The apparatus is used to fractionate secondary cellulose acetates with weight average molecular weights of *ca.* 35 000 and 60 000. Fifty fractions were isolated which increase in intrinsic viscosity with fraction number. The steady increase of intrinsic viscosity with fraction number appears to preclude effects from variations in acetyl substitution on the fractionation. Recoveries above 90 % representative of the entire sample, are demonstrated.

The effect of molecular weight, shaking time and concentration on the diffusion of cellulose acetate from acetone solutions into charcoal is illustrated by means of single batch experiments. A molecular weight gradient is demonstrated in the capillaries of the charcoal. Preliminary intrinsic viscosity weight distribution diagrams are presented.

A method for determining the intrinsic viscosity of cellulose acetate in acetone by single measurements is given. Graphs of $c\,[\eta]$ *vs.* t, where c is concentration, $[\eta]$ the intrinsic viscosity and t the efflux time of the solution in seconds, were obtained on several Ostwald pipettes [11] of low kinetic energy error. The graphs are based on Martins [12] equation $\log(\eta_{sp}/c) = \log[\eta] + K[\eta]\,c$, rearranged to the form $\eta_{sp} = c\,[\eta] \cdot 10^{Kc[\eta]} = \frac{t-t_0}{t_0}$ where t_0 is efflux time of the solvent. The K value was found to be 0.21 on secondary cellulose acetates ranging in intrinsic viscosity from 0.95 to 2.24, in concentrations up to 0.35 %.

Diffusion methods for the preparative fractionation of high molecular weight compounds have not been successfully used. Suitable membranes have not been found and when columns of porous media are used, selective migration is difficult to realize. As far as is known, capillary systems which take advantage of the variation in diffusion constant with molecular weight have not been employed for preparative polymer fractionation.

* American-Scandinavian Foundation Fellow 1953.

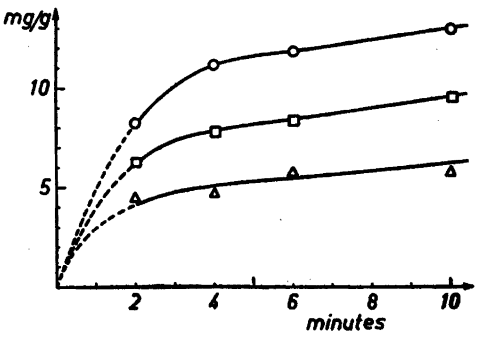

Fig. 1. The uptake versus time of cellulose acetate samples of varying molecular weight by charcoal. Circles: sample 0.95, squares: sample 1.62, triangles: sample 2.24.

When charcoal has been used for the chromatography of cellulose derivatives [1-4] equilibrium conditions have been sought but were difficult to obtain although slow flow rates were used. Nonquantitative elution has been the main difficulty hindering the use of chromatography as a practical fractionation method for high polymers.

The effect of diffusion upon the chromatography of cellulose derivatives and other polymers with charcoal has been noted by Claesson [5] who found that above a molecular weight of about 10 000, decreased amounts were taken up by the charcoal with increasing molecular weight, reflecting the decrease in diffusion constant.

Diffusion effects were not evident when starch was used by Brooks and Badger [6] for separations of cellulose nitrate. The rapid equilibrium observed, the preferential uptake of the larger molecules and the marked dependence of the separation on the degree of nitrate substitution indicate that adsorption was the single affecting factor.

The fractionation of polymethyl-siloxanes by gradient elution from charcoal has recently been reported [7]. An increase in molecular weight with increasing eluent was found.

In the method described in this paper, the preferential diffusion of lower weight molecules into porous charcoal is used to effect cellulose acetate fractionation. Adsorption influences the separation by retaining the more deeply diffused material.

The effect of time and of varying molecular weights on the diffusion into charcoal

Three secondary cellulose acetate samples with intrinsic viscosities of 2.24, 1.62 and 0.95 were used which are identified in the following text by their intrinsic viscosity values. Information about the materials used and further experimental details are found in the Experimental part.

Solutions containing 300 mg of the 2.24, 1.62 and 0.95 samples in 15 ml acetone were shaken for 2, 4, 6 and 10 minutes with six grams of acetone-saturated charcoal.

After decantation the charcoal was first washed for 30 seconds and then for ten minutes successively with 25 ml portions of acetone. The total material recovered in this way was then subtracted from the original sample weight to obtain the uptake per gram charcoal. The results are shown in Fig. 1.

The rapidity of the diffusion into charcoal and the pronounced dependence of the uptake on molecular weight is seen. The decreased rate of uptake after about four minutes followed by a nearly linear uptake by the charcoal during extended shaking periods is generally found in all experiments.

The preferential removal of lower weight molecules by charcoal

Solutions of the three samples of cellulose acetate, each in 25 ml acetone, 750 mg in four cases and 500 mg in another, were shaken for five minutes with ten grams of acetone saturated charcoal. After decanting the original solution the charcoal was shaken with successive 25 ml portions of acetone for five minutes until measurable acetate was no longer found. The charcoal was then shaken further with successive 25 ml volumes of dioxan. The amounts and intrinsic viscosities of the acetate first decanted and of that obtained from each of the successive washings and elutions were then measured.

The analytical data, given in Table 1, show the removal of successively lower molecular weight portions of the sample distribution by the repeated washings, evidence of a molecular weight gradient in the capillaries of the charcoal, the smallest molecules having penetrated most deeply. The intrinsic viscosities of the material obtained after prolonged shaking with dioxan evidently represent an average of the cellulose acetate remaining in the charcoal after no more material could be removed by the acetone washings.

That a gradient exists as well in the lower molecular weight material left in the charcoal after the acetone washings, is indicated in one column in Table 1 where the charcoal was shaken with dioxan for successive five minute periods.

The intrinsic viscosity weight average of the recovered fractions is lower than that of the original sample. This appeared to be due to analytical difficulties rather than to the holdup of high molecular weight material.

The effect of prolonged shakings and varying concentrations of cellulose acetate on the uptake

The results are shown in Table 2 which gives analyses of the solutions decanted after shaking solutions of sample 1.62 with the charcoal. Increased uptake with increased concentration is found as predicted by diffusion theory.

The increased uptake with time is reflected by the increasing intrinsic viscosity values.

THE SOLID PHASE COUNTERCURRENT APPARATUS

Although a machine was not developed for its use, Craig and co-workers [8] suggested that a solid could be used in countercurrent separations. To con-

Table 1. The effect of successive washings of the charcoal with solvent and eluent after shaking cellulose acetate solutions with charcoal for five minutes. a: original sample in 25 ml acetone. Acetone washes five minutes, dioxan washes two hours or more except where superscript b indicates five minutes shaking.

	[η]	Found mg	[η]	Found mg	[η]	Found mg	[η]	Found mg	[η]	Found mg
Original sample	0.95	750[a]	1.62	750[a]	1.62	750[a]	2.24	500[a]	2.24	750[a]
First decantation	0.96	375	1.61	344	1.64	452	2.33	218	2.27	355
Acetone washes:										
1.	0.98	132	1.52	203	1.65	157	2.30	149	2.44	168
2.	0.94	29	1.54	42	1.65	33	2.12	26	2.42	39
3.	0.76	20	1.31	21	1.48	9		10	2.42	23
Remaining in charcoal		194		140		99		97		165
Dioxane elutions:										
1.	0.48	91	0.86	65	0.69[b]	43[b]	1.30	44	1.48	52
2.	0.60	29	0.85	21	0.64[b]	22[b]	1.21	17	1.29	23
3.	0.48	10	0.78	11	0.53[b]	8[b]	1.20	6	1.63	9
4.					1.09	21				
Remaining in charcoal		64		43		5		30		81
Intrinsic viscosity weight average of recovered fractions	0.86	686	1.52	707	1.53	745	2.15	470	2.22	669

struct an apparatus in which a solid is used as the stationary phase, modifications of the Craig [9] apparatus design were made.

A fifty tube apparatus, blown as a single piece, is seen in Fig. 2. Since decanting rather than separation at the contact level of two liquid phases is involved, and maximum capacity is desirable, the outlet is placed as high as possible on the mixing tube. The transfer reservoirs, best seen in the closeup

Table 2. Effect of concentration and prolonged shaking times on the uptake of cellulose acetate 1.62 and on the intrinsic viscosity of the decanted solutions. Four grams charcoal shaken with each solution. a: 600 mg and b: 135 mg present in 20, 30 and 45 ml acetone, respectively.

% Conc.	Uptake by charcoal mg/g					Intrinsic viscosity				
	Shaking time, minutes					Shaking time, minutes				
	4	15	30	60	600	4	15	30	60	600
3.0[a]	32.5	36.2	40.0	43.0	48.5	1.71	1.71	1.75	1.75	1.85
2.0[a]	20.0	24.5	27.3	33.2	35.5	1.67	1.71	1.76	1.79	1.83
1.33[a]	15.0	16.3	17.3	21.8	25.0	1.67	1.68	1.68	1.77	1.78
0.68[b]	13.3	10.0	13.3	16.7	18.0	1.85	2.11	1.95	1.86	2.15
0.45[b]	11.2	12.8	10.0	14.5	16.8	1.82	1.85	1.80	1.99	2.10
0.30[b]	8.8	11.0	11.3	11.5	17.0	1.83		1.88	1.94	2.05

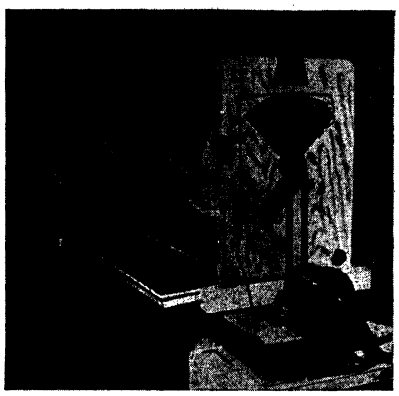

Fig. 2. The solid phase countercurrent apparatus.

in Fig. 3, have a powder puff shape, which provides good capacity as well as compactness. The volume of the mixing tubes from bottom to outlet, is 50 ml and that of the transfer reservoirs 65 ml.

The reversing motor used for shaking, seen in Fig. 2, has a speed of 4.5 revs. per minute. The extent of tipping is controlled by a rod extending from

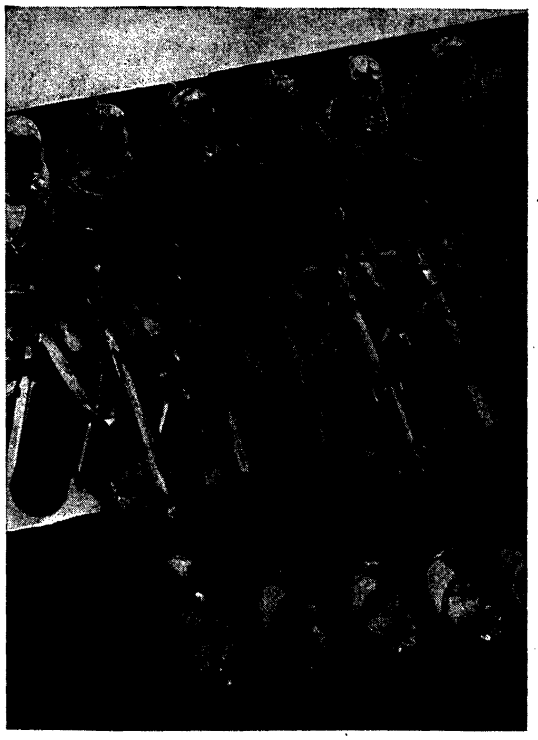

Fig. 3. Closeup of apparatus. Solution transfer is from right to left.

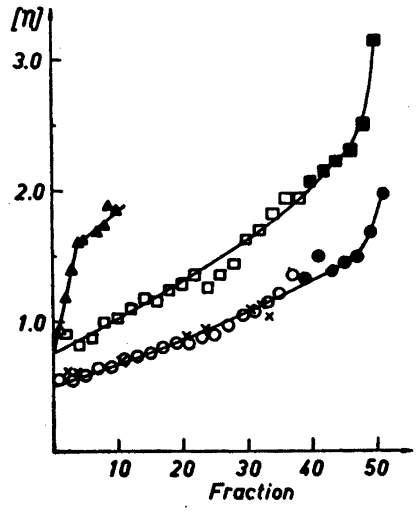

Fig. 4. Intrinsic viscosity versus fraction number. Triangles: five tube run with acetate sample 1.62. Squares: every second fraction from fifty tube run with sample 1.62. Circles: every second fraction from fifty tube run with sample 0.95. The solid symbols indicate the decanted acetone fractions.

the motor shaft to adjustable micro switches. Effective, though gentle shaking is obtained by this arrangement. The shaking time is controlled by a timer wired into the relay. Decanting, transfer and additions of solvent are done by hand.

Five tube countercurrent run

Five grams of charcoal, put into each of the first five tubes of the apparatus, were washed twice with acetone. Fifty ml of acetone containing 2.5 g of the 1.62 cellulose acetate was introduced into the first tube. After four minutes shaking and decantation, 25 ml acetone was added from the first reservoir to the first tube, while the decanted solution automatically transferred to the second tube. Acetone was added in this way four times following each four minute shaking period. At the end of twenty minutes the remainder of the original solution was thus decanted from the fifth mixing tube. Elution was done by shaking the charcoal with 25, 15 and 10 ml volumes successively, of dioxan, for one hour or more. Ten fractions resulted; five decanted to the reservoirs and five obtained by the subsequent elution of the charcoal. For clarity "decanted fractions" will refer to the solutions decanted before the dioxan elution.

Concentrations were determined on all the fractions and intrinsic viscosities on the first elution and on the decanted fractions. 97 % of the acetate was recovered, 20 % by elution from the charcoal.

The degree of separation obtained is shown by the intrinsic viscosity values in Fig. 4. The intrinsic viscosity range, from 0.9 to 1.8, after the short run, illustrates how rapidly the separation is initiated.

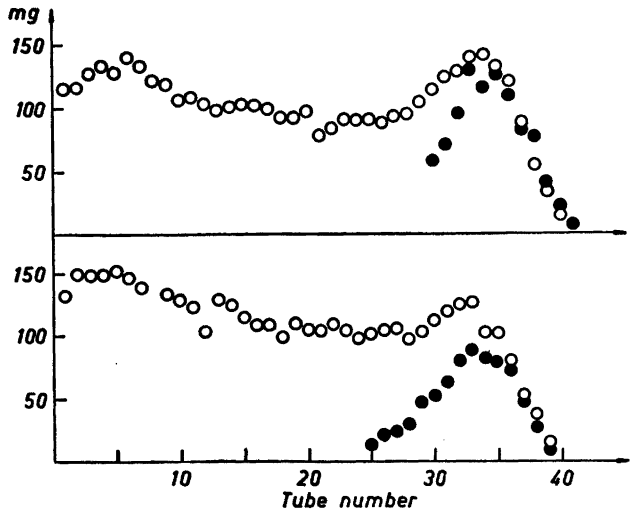

Fig. 5. Fraction weight versus tube number. Upper: from fifty tube run with cellulose acetate sample 1.62. Lower: from fifty tube run with sample 0.95. The solid symbols indicate the decanted acetone fractions.

Fifty tube countercurrent run with low molecular weight cellulose acetate

Nine grams of charcoal used per tube was first washed with acetone. Five grams of the 0.95 acetate sample in 70 ml acetone was introduced in two 35 ml portions to the first two tubes as the high viscosity of the full charge when used in the limited volume available in the one tube resulted in poor mixing. The cellulose acetate solution was followed by 25 ml additions of acetone to the first charcoal stage. Five minute shaking periods were used between the successive additions of solvent.

Elution was first done by shaking the charcoal successively with 25 and 15 ml dioxan for two hours or more. 15 % of the original material was in the decanted fractions which, with the eluted material from the two shakings with dioxan, accounted for 66 % of the original sample. The intrinsic viscosities, determined on every second fraction, are shown in Fig. 4.

A third elution was done with 25 ml dioxan. When this material was added to that already obtained, 92 % had been recovered. The viscosities determined on combined, contiguous pairs from this final elution are indicated by crosses on the curve in Fig. 4. They coincide well with the viscosity curve resulting from the previous elutions, showing that after the prolonged shaking with dioxan no molecular weight gradient has been detected. It was also found that the last elution removed nearly like proportions of material regardless of DP, attesting to the effectiveness of the elution.

The weight of each fraction is shown in the lower portion of Fig. 5. It can be seen from the figure that the higher viscosity decanted material

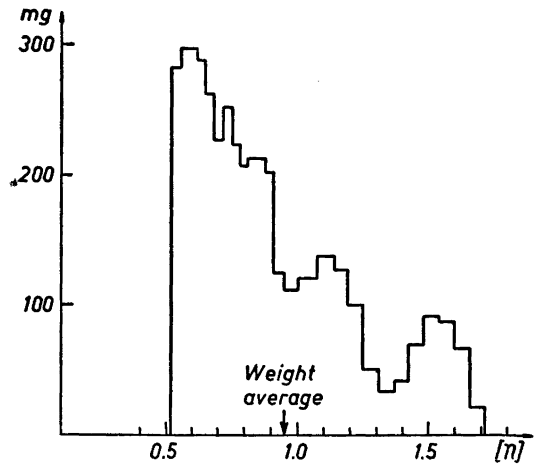

Fig. 6. The intrinsic viscosity weight distribution of the fractions from fifty tube run with acetate sample 0.95.

appears at tube 25, while beyond tube 40, extending to tube 50, acetate is found neither in the charcoal nor in the decanted acetone, illustrating the countercurrent effect.

The intrinsic viscosity weight distribution is given in Fig. 6. It is based on 100 % recovery, assuming that the material eluted was representative of that remaining in the charcoal. To prepare the diagram, the fraction weights were used directly. The intrinsic viscosity values however, were taken from a curve drawn by eye through the viscosity points in Fig. 4. The weight average intrinsic viscosity is the same as that of the original sample, confirming the reliability of the viscosity method used and the effectiveness of the recovery.

Countercurrent run with higher DP cellulose acetate

5 g of the 1.62 acetate in 70 ml acetone were introduced to the first two tubes of the apparatus. Other conditions were the same as those used with the lower DP sample.

25 % was found in the decanted fractions in comparison to 15 % when the lower DP acetate was run under like conditions. When this material was added to that obtained in the elutions with dioxan, 60 % was recovered. No further elution was done. The intrinsic viscositiy of each second fraction is shown in Fig. 4 and the amounts recovered, in Fig. 5.

The intrinsic viscosity weight distribution, shown in Fig. 7 is based on 100 % recovery. The intrinsic viscosity weight average, 1.53, is lower than that of the original sample. This may be due to unrepresentative elution or analytical errors.

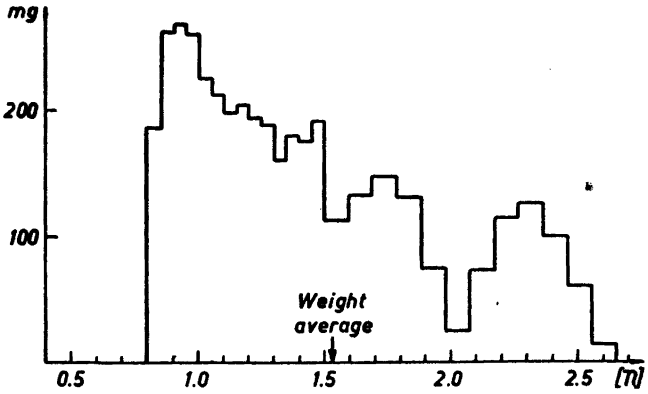

Fig. 7. The intrinsic viscosity weight distribution of the fractions from fifty tube run with acetate sample 1.62.

EXPERIMENTAL

Materials. The cellulose acetates are Hercules Powder Company products. The samples which have intrinsic viscosities of 2.24, 1.62 and 0.95 — weight average molecular weights of *ca.* 83 000, 60 000 and 35 000 — are substituted to the extent of 2.45, 2.39 and 2.48, respectively.

The charcoal, a Norit Company product, is a rather soft, activated carbon with a particle diameter of 0.7 mm. Acetone of analytical purity was used throughout. The dioxan used had 99 % purity and was used without further treatment.

Experimental details. To wash the charcoal, 25 ml portions of acetone were added simultaneously from the reservoirs and shaken with the charcoal for fifteen minutes. This was done twice. In addition to removing excessive charcoal fines, the acetone remaining on the charcoal after washing, served to dilute the cellulose acetate solution making it possible to transfer the original charge through the apparatus. Similar diffusion effects were found whether the cellulose acetate solution was shaken with dry charcoal or with acetone-saturated charcoal.

After the run the decanted acetone fractions in the reservoirs were removed with a syringe and centrifuged to remove charcoal fines. To determine concentrations, aliquots were precipitated in water on a steam bath in small, thin drying phials. The phials were hung in a vertical rack in an electrically heated drying column [10] under vacuum for twenty minutes. During the last ten minutes of this period 105—108° C was maintained. Viscosities were determined directly on the decanted acetone fractions after adjustment of concentrations.

For elution, the dioxan was added simultaneously to the charcoal from all the reservoirs and generally shaken two hours or more. Aliquots of the dioxan solutions were precipitated and dried in the same manner as the acetone fractions. The dried samples were dispersed in acetone for the viscosity determination.

Viscosity Measurements. Intrinsic viscosity analyses are laborious and since small quantities were analyzed, measurements at several concentrations would have been very difficult. In consequence means were sought to obtain the value from one efflux time at any single concentration in a low concentration range.

Ostwald pipettes [11] recommended for a range between 0.3 and 5.0 centipoises were used at 25° C ± 0.02. 2 ml solution was required. Conventional semi-log plots of spec. viscosity / conc. *vs.* conc. were made on several secondary cellulose acetates and on mixtures of these.

The finding of Martin [12] that the slopes obtained have a linear relation to their limiting viscosity values at zero concentrations was confirmed in concentrations up to 0.35 %

on samples ranging in intrinsic viscosity between 0.95 and 2.24. The low kinetic energy error of the pipettes was found to be a necessary condition in determining the relation.

A K value of 0.21 was obtained by plotting the slopes against the corresponding intrinsic viscosities. This was then used in Martins expression

$$\log \left(\frac{\eta_{sp}}{c}\right) = \log [\eta] + K [\eta] \cdot c$$

rearranged to the form

$$\eta_{sp} = c \cdot [\eta] \ 10^{Kc[\eta]} = \frac{t - t_0}{t_0}$$

where t, is efflux time of the solution, t_0, that of the solvent, c, the concentration and $[\eta]$, the intrinsic viscosity.

By using the equation in this form, graphs of $c \cdot [\eta]$ *vs.* t were obtained for several pipettes. One efflux time in the appropriate concentration range is thus sufficient. The $c \cdot [\eta]$ value found on the other axis need only be read off and divided by the concentration used.

DISCUSSION

An important aspect of the countercurrent method is the ease with which larger capacities and quantities can be employed. The rapidity of the fractionation is an obvious advantage.

It appears that a single batch separation method could be refined and used to partially characterize the distribution. It is also a convenient means to obtain a preliminary partition of the sample which can then be followed by the countercurrent fractionation of the upper or lower molecular weight segment.

In the countercurrent runs, the smaller molecules, continually pressing into the capillaries of the charcoal at each stage of the moving system, had an opportunity to be adsorbed. The decanted fractions of higher DP, were selectively refused by the charcoal stages, resulting in the progressively increasing intrinsic viscosity of the decanted, as well as the eluted, fractions.

When extended shaking times were used in a run with acetate 1.62, a small percentage of the sample, above viscosity 2.5, failed to diffuse into the charcoal. There was also some evidence that representative elution was more difficult after the extended contact between the charcoal and the cellulose acetate solution.

The steady increase of intrinsic viscosity with fraction number seems to preclude much effect from variations in the degree of substitution on the fractionation. Acetyl analyses are planned on the fractions.

The distributions obtained are asymmetrical with positive skewness conforming with the preponderance of theoretical and experimental evidence[13,14]. The presence of more than one maximum in the weight distribution diagrams is believed to have resulted from the experimental conditions. Homogeneity measurements of the fractions are planned.

It appears likely that other polymers may be separated with the demonstrated countercurrent method and such experiments are in progress.

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The author is particularly indebted to Professor Börje Steenberg for proposing the project, his enthusiastic interest, the generous provision of facilities and for editing the

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The assistance of Miss Inger Almsäter and Miss Elizabeth Johansson has been invaluable. Dr. A. F. Martin, Hercules Powder Company, kindly provided the cellulose acetate samples with accompanying analytical data.

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Determination of Mercury in Urine

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Existing methods for the determination of mercury in urine are rather time-consuming. A more rapid method has therefore been elaborated, involving a wet combustion with sulphuric acid and potassium permanganate. After reduction of the excess of permanganate with hydrogen peroxide and oxalic acid the mercury is extracted with dithizone solution at a pH value of between 0 and 1, where other metals do not interfere. The dithizone-mercury dithizonate solution formed is divided in two parts, one of which is extracted with sodium thiosulphate solution and then used as blank solution in the spectrophotometric determination of the dithizonate in the other part. The method has been used for some years as routine control for persons employed in the manufacture of methyl mercuric hydroxide and in steeping seed with mercury containing material.

The method most frequently used in Sweden for the determination of mercury in urine and biological material is that of Vesterberg and Sjöholm[1]. According to this method the sample is subjected to wet combustion in an acidic solution of chlorine and, after evaporation of the chlorine, mercury is precipitated as sulphide with cadmium sulphide as collector. The mixture of sulphides is filtered off, dissolved in a solution of potassium chlorate and hydrochloric acid and reprecipitated. After treatment again with chlorine and hydrochloric acid, followed by evaporation of the chlorine, the mercury is extracted with a solution of dithizone and the excess of the reagent titrated with a silver nitrate solution. This method is time consuming and therefore not suited for routine analysis; the result of an analysis is only obtained after 4—5 days. For some years another more rapid method of analysis has been used in this laboratory with good results. It is much less laborious and the results are available after about 12 hours. In this method also the sample is subjected to wet combustion and the mercury determined by means of dithizone. The method does not contain anything essentially new but is the result of a critical review of available procedures. It has been used only for the routine control of persons, employed in the manufacture of methyl mercuric hydroxide and in steeping seed with mercury containing material. The method may possibly be useful in other cases and is published for that reason.

The determination of mercury in organic material involves mainly three problems, namely the oxidation of the organic material without the loss of mercury, reduction of the oxidising agents used and finally the actual determination of mercury. According to the proposed method the sample is oxidised in an acid potassium permanganate solution[2], which was found to be the most rapid method. No loss of mercury has been observed in any case with the types of mercury compounds used. For destruction of the excess permanganate many reducing agents have been proposed such as hydroxylamine and hydrazine. However when these substances are used, the mercury can not be extracted quantitatively with dithizone and the results of the analyses vary [3]. The best results have been obtained by reduction of the permanganate with a slight excess of hydrogen peroxide followed by the addition of a small amount of permanganate and finally decolourising with oxalic acid. Using this procedure it very occasionally happens that the dithizone is destroyed, probably by oxidation. If the sample solution after oxidation is left until the next day this phenomenon usually disappears. In order to avoid the loss of the whole analysis a test extraction is made with a small part of the sample solution with dithizone solution. If this solution turns a pale green or yellow green colour distinctly different from the usual green to olive green colour of the dithizone-dithizonate mixture or if the dithizone solution fades completely, the sample solution is boiled with some hydrogen peroxide after oxidation of the excess oxalic acid by the addition of permanganate. The oxalic acid must be destroyed to avoid the loss of mercury during boiling.

The solution of inorganic mercury thus obtained is extracted with a solution of dithizone in chloroform. To avoid interference from other metals that may be present, the extraction is carried out at a pH-value of between 0 and 1 [4,5], which is most easily obtained by diluting the solution of the sample. The spectrophotometric determination of the mercury may be done in two ways, either by determining the decrease in the extinction of the dithizone-solution at 520 mμ, the highest absorption maximum of the dithizone, or by determining the increase of the extinction at 490 mμ, the absorption maximum of the mercury dithizonate. The latter method seems to be the best for the small quantities of mercury usually occurring in urine. The original dithizone solution may be used as reference solution. A slightly more exact result will be obtained by extracting the mercury from part of the solution to be determined, and using the dithizone solution thus obtained as a reference solution. In this way an automatic correction for the eventual fading of the dithizone will be obtained and as well for other heavy metals that may occasionally be present.

Solutions of potassium iodide [5] or of sodium thiosulphate [2], both giving good results, have been suggested for the extraction of mercury.

PROCEDURE

Reagents

1) Concentrated sulphuric acid
2) Potassium permanganate
3) Hydrogen peroxide 15 % (1 volume hydrogen peroxide 30 % + 1 volume water).
4) Potassium permanganate solution in water (4 %).
5) Oxalic acid solution 0.5 *M*.

Table 1. Recovery of added amounts of mercury. A comparison of the amount (in μg) of mercury added with that found by analysis.

a) *Methyl mercuric hydroxide*		
Added	Found	Difference
10	10	0
10	10	0
20	17	−3
20	21	+1
30	31	+1
30	32	+2
40	42	+2
40	42	+2
50	45	−5
50	45	−5

b) *Phenyl mercuric acetate*		
Added	Found	Difference
20	23	+3
20	24	+4

c) *Mercuric chloride*		
Added	Found	Difference
0	0	0
0	0	0
20	19	−1
30	29	−1
0	0	0
0	0	0
0	0	0
19	20	+1
30	31	+1
25	25	0
14	13	−1
20	19	−1
0	0	0
0	0	0
20	20	0
10	9	−1
10	9	−1
15	16	+1

6) Hydrogen peroxide 5 % (1 volume hydrogen peroxide 30 % + 5 volumes water).
7) Dithizone solution (10 mg of dithizone are dissolved in chloroform and diluted to 1 000 ml).
8) Sulphuric acid 0.1 *N*.
9) Sodium thiosulphate solution in water (1.5 %).
10) Mercury standard solution I (100 μg Hg^{2+} per ml). Mercuric chloride (135.7 mg) is dissolved in water and diluted to 1 000 ml.
11) Mercury standard solution II (2 μg Hg^{2+} per ml). Mercury standard solution I (5.00 ml) is diluted with water to 250 ml. This must be made immediately before use.

Destruction of organic matter. Shake the sample of urine vigorously in order to disperse any sediment and pour 500 ml into a 2 000 ml flask. Add 50 ml of conc. sulphuric acid in portions and then 25 g of solid potassium permanganate and shake the flask gently a couple of times. Heat over a flame under reflux, until boiling, and continue boiling for 8 hrs. If all the potassium permanganate is consumed during this period, cool the sample to room temperature and add about 10 g of solid potassium permanganate. Continue the boiling for a total time of 8 hrs. Cool to about 20° C. Wash the condenser down into the flask with about 15 ml of dist. water. Add hydrogen peroxide, 15 % in portions of 2—3 ml, each time shaking the flask several times rather vigorously until the sample is decolourised. Add a small excess of potassium permanganate solution and decolourise again, but this time with oxalic acid solution, and then add an excess of 2—3 ml of the oxalic acid solution.

Preliminary test extraction. Measure the volume of the decolourised sample in a cylinder and then transfer 20 ml to a 50 ml separating funnel. Add 1 ml dithizone solution and shake vigorously several times. Examine the dithizone layer by a diffuse light. If the dithizone solution fades, pour the main solution back into the flask and add a small excess of potassium permanganate solution and then decolourise with hydrogen peroxide solution, 5 %. After addition of an excess (2 ml) of hydrogen peroxide, reflux the sample for an hour and it will after cooling, be ready for further treatment. If the dithizone did not fade in the preliminary test extraction, this reheating is unnecessary.

Extraction of the mercury. After dilution of the sample solution to 1.6 l, pour it into a 2 l separating funnel and add 25 ml dithizone solution with a pipette. Shake the separating funnel vigorously 100 times, and separate the chloroform layer and filter it through glass wool into a 50 ml flask. Use part of this solution as the sample for the measurement of the extinction while the rest is poured into a 100 ml separating funnel and shaken with a mixture of 50 ml 0.1 *N* sulphuric acid and 10 ml 1.5 % sodium thiosulphate. This mixture is previously saturated by shaking with some chloroform which is then carefully separated. After shaking with the thiosulphate solution the dithizone solution should have a clearly bluish green colour and after filtration through glass wool it is used as the blank solution for the absorption measurement. This is made at a wave length of 490 mμ and the value obtained is compared with a standard curve, made using solutions of known concentration. In the calculation of the quantity of mercury in the sample correction must be made for traces of mercury in the chemicals used and for the quantity of the sample consumed by the preliminary test extraction.

Standard curve. A standard curve is made by mixing 1.6 l water and 50 ml conc. sulphuric acid with different known quantities of mercury standard II. It is convenient to use 0, 5, 10, 15, 20 and 25 ml equivalent to 0, 10, 20, 30, 40 and 50 micrograms of mercury. The samples are shaken with 25 ml dithizone solution which is then treated in the same way as before, and the standard curve is made with the extinction values obtained.

Test analyses. As well as the analyses done during the elaboration of this method, check analyses have also been carried out later while the method has been in use. To urine or in some cases to water has been added known quantities of mercuric chloride, methyl mercuric hydroxide or phenyl mercuric acetate and these samples have been mixed with the routine analyses. In Table 1 all the samples are collected that have been analysed during one period of time. As is clear from the table the difference between the mercury added and that found amounts to only a few micrograms, a fact that must be considered satisfying.

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21-Derivatives of Compound S, Cortisone and Hydrocortisone

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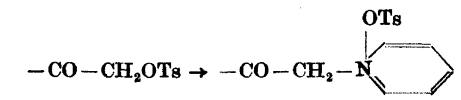
Treatment of compound S, cortisone and hydrocortisone with tosyl chloride and pyridine at low temperature (+ 2° and − 18°) affords the corresponding 21-tosylates. By means of tosyl anhydride and pyridine the 21-tosylate of compound S and cortisone have been prepared at higher temperature (*e. g.* room temperature). Cortisone tosylate has been prepared at room temperature by treatment with tosyl chloride and NaOH-solution. By reaction with salts of halide acids (NaI, NaBr, $CaCl_2$ and NH_4Cl) the corresponding halogen compounds have been prepared from the 21-tosylates. Treatment of the 21-iodo compounds with KCN affords the 21-cyano compounds. From the 21-tosylates and the 21-iodo compounds the 21-thiocyanates and the 21-thiolacetates have been prepared by reaction with KSCN and potassium thiolacetate, respectively.

For the purpose of investigating the biological effect of steroids of the cortisone (II) and hydrocortisone (III) type in which the primary hydroxyl group in the 21-position has been replaced by other groups, in particular groups containing S or N atoms, it would be desirable from II or III to prepare the corresponding steroids in which a halogen atom has been substituted for the hydroxyl group in the 21-position.

One of the generally known methods of substituting a halogen atom for a primary OH group is to prepare the *p*-toluenesulphonic acid ester (the tosylate) and subsequently treat the tosylate with a salt of a halide acid [1].

The attempts hitherto made to obtain the tosylate in the 21-position in pure condition and in good yield by treating steroids with the side chain —CO—CH_2OH with tosyl chloride in the presence of pyridine have not succeeded.

Reichstein and Schindler [2] recommend the preparation of such tosylates from the corresponding diazo-ketone, —$COCHN_2$, by treatment with anhydrous *p*-toluenesulphonic acid, as the tosylate formed primarily through the interaction of the 21-OH group with tosyl chloride in pyridine will readily react with pyridine to form the pyridinium salt:



Reichstein and Fuchs[3] have treated desoxycorticosterone and corticosterone in pyridine/chloroform solution with tosyl chloride at room temperature and have obtained a mixture of tosylate and 21-chloro compound and a small amount of pyridinium compound. A considerable excess of pyridine was avoided so that only a small amount of pyridinium compound was formed, but the tosylate formed primarily will react to some extent with pyridine hydrochloride to form the 21-chloro compound:

$$—CO—CH_2OTs \rightarrow —CO—CH_2Cl$$

Djerassi and Nussbaum[4] have treated compound S (I) and cortisone, respectively, in pyridine/chloroform solution at 0° C with tosyl chloride allowing the solution to stand at room temperature for 14 and 17 hours. From compound S a yield of 45 % of a substance which consists chiefly of the 21-chloro compound is obtained. From cortisone a yield of 65 % is obtained of a substance which probably contains pyridinium salt, 21-chloro compound, and tosylate.

Leanza and coworkers[5] have treated cortisone in pure pyridine solution with tosyl chloride heating gently for a few minutes and then allowing the solution to stand overnight at room temperature. In one case they have obtained 78.5 % 21-pyridinium chloride and in another 56 % 21-pyridinium tosylate. In a third case the cortisone solution was cooled to 15° C, tosyl chloride was added, the mixture was kept at a temperature of 10—15° C for one hour and allowed to stand overnight at room temperature. This resulted in a yield of 46 % of 21-chloro compound.

If the side reactions are taken to be the result of reactions between the tosylate formed primarily and the other components in the solution, tosylation must be assumed to be a reaction which takes place very readily. None of the authors mentioned apply temperatures below 0° C, and the possibility therefore existed that at very low temperatures tosylation would occur while the side reactions would take place less readily.

It proved possible to prepare the 21-tosylates of compound S (IV), cortisone (V), and hydrocortisone (VI) in pure condition and in good yields (in the case of cortisone tosylate (V) the yield was, *e. g.*, 73 %) by dissolving the steroid in pure, dry pyridine, cooling the solution in dry ice-acetone, adding tosyl chloride dissolved in dry methylene chloride and cooled to such an extent that the tosyl chloride just remained in solution, allowing the mixture to stand in dry ice-acetone for about two hours while occasionally shaking the flask, and finally setting aside the solution for 16—17 hours at +2° C and —18° C, respectively.

The preparation of cortisone tosylate (V) requires cooling to a lower temperature than does the preparation of compound S tosylate (IV). If the same temperature is used as in the preparation of compound S tosylate (IV), a substance is formed which has a melting point of 230—232° C and contains 7.1 % chlorine. As the Lassaigne test for N is negative (*i. e.* it is not a pyridinium compound), the substance must be assumed to consist of about 75 % 21-chloro compound (the theoretical content of chlorine in the 21-chloro compound is 9.36 %).

To eliminate this formation of 21-chloro compound completely it was tried to use *p*-toluenesulphonic acid anhydride (tosyl anhydride) [6] instead of tosyl chloride. Compound S tosylate (IV) was prepared by means of tosyl anhydride in pyridine and methylene chloride at low temperature, room temperature and with refluxing. The yields obtained at low temperature and at room temperature were the same, although somewhat lower than those obtained by the tosyl chloride method. When refluxing, the yields obtained were very low, as the formation of pyridinium salt is of course facilitated in this case, but in spite of this it is possible to isolate the tosylate in pure condition. As regards the preparation of cortisone tosylate (V) the same conditions apply when using tosyl anhydride as when using tosyl chloride. Already at room temperature the yield is low, the formation of side products occurring more readily than in the case of the compound S tosylate (IV) preparation.

To eliminate the formation of pyridinium salts and 21-chloro compound an attempt was made to replace pyridine with aqueous NaOH solution; cortisone tosylate (V) was obtained in good yield at a temperature of 25° C when using dioxan as the solvent, adding tosyl chloride and adding NaOH solution dropwise.

The tosylates tend to crystallize with 1 mole methanol, benzene, or ethanol which cannot be removed readily by drying. In case of cortisone tosylate (V) the benzene does not disappear when subjected to vacuum drying (about 10^{-1} mm) at room temperature for 20 hours. For this reason the melting points are often somewhat indefinite, and the melting is accompanied by vesiculation.

By means of NaI in acetone the corresponding 21-iodo compounds Δ^4-21-iodopregnene-3,20-dione-17α-ol (VII), Δ^4-21-iodopregnene-3,11,20-trione-17α-ol (VIII), and Δ^4-21-iodopregnene-3,20-dione-11β,17α-diol (IX) were prepared from compound S tosylate (IV), cortisone tosylate (V), and hydrocortisone tosylate (VI). These iodo compounds are white crystalline substances, which are fairly stable. After storage for about one month without exposure to light they tend to turn dark.

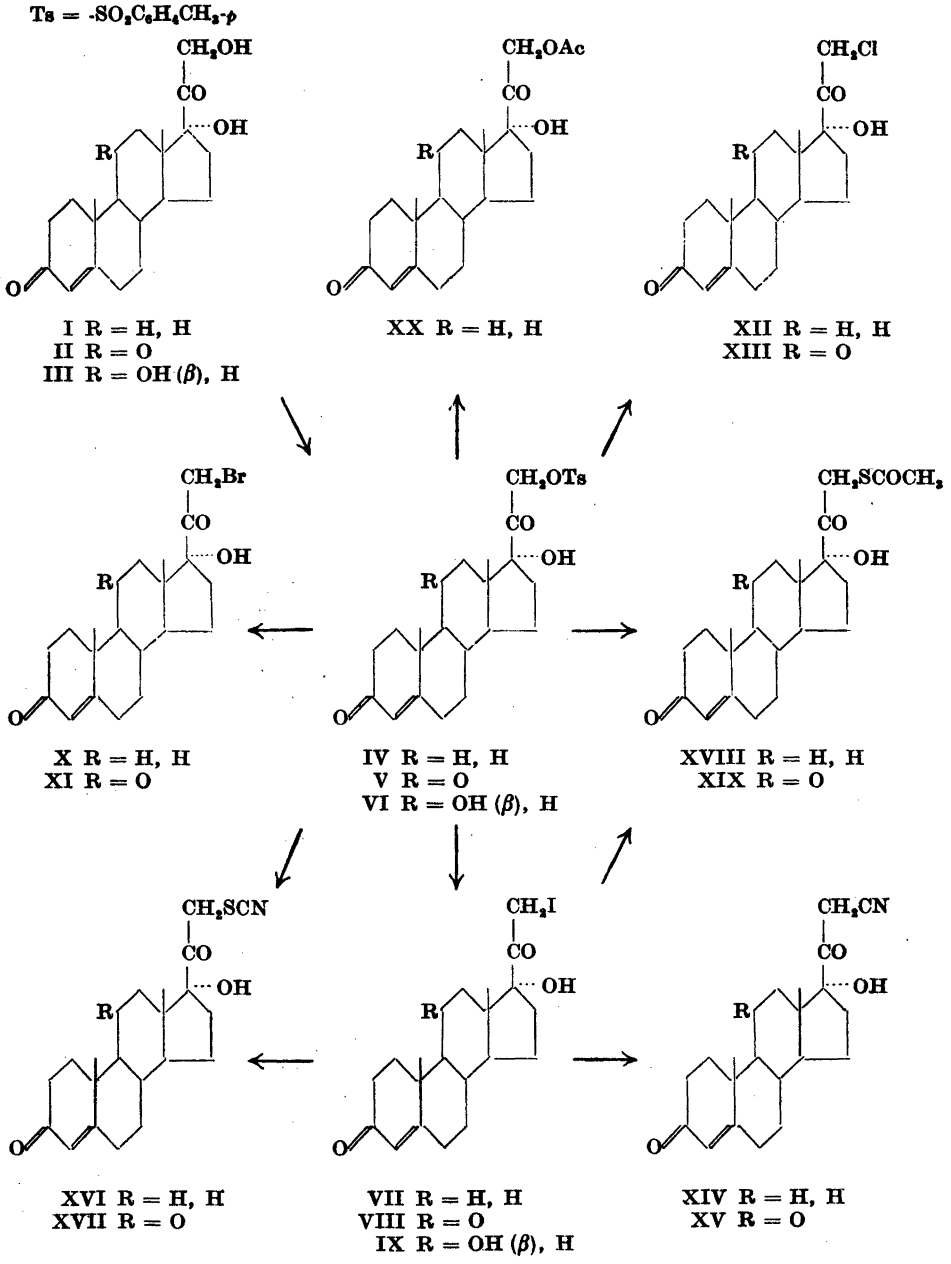
By the action of NaBr in methanol the 21-bromo compounds Δ^4-21-bromopregnene-3,20-dione-17α-ol (X) and Δ^4-21-bromopregnene-3,11,20-trione-17α-ol (XI) were prepared from IV and V.

According to Reichstein and Schindler [2] NaCl cannot be used for the preparation of 21-chloro compounds from tosylates on account of too low solubility in acetone and methanol, for which reason these authors use tetramethylammonium chloride.

Since $CaCl_2$ and NH_4Cl are soluble in ethanol, these substances were used for the preparation of the 21-chloro compounds Δ^4-21-chloropregnene-3,20-dione-17α-ol (XII) and Δ^4-21-chloropregnene-3,11,20-trione-17α-ol (XIII) from compound S tosylate (IV) and cortisone tosylate (V).

From the 21-iodo compounds (VII) and (VIII) the 21-cyano compounds, Δ^4-21-cyanopregnene-3,20-dione-17α-ol (XIV) and Δ^4-21-cyanopregnene-3,11,20-trione-17α-ol (XV), were prepared by means of KCN in methanol. By means of KSCN in acetone the 21-thiocyano compounds, Δ^4-21-thiocyanopregnene-3,20-dione-17α-ol (XVI) and Δ^4-21-thiocyanopregnene-3,11,20-trione-17α-ol (XVII), were prepared. XVII was also prepared from cortisone tosylate (V) by reaction with KSCN in acetone.

For the identification of the 21-halogen compounds and the 21-tosylates, the thiolacetates, compound S-thiolacetate (Δ^4-pregnene-3,20-dione-17α-ol-21-thiol-21-acetate, XVIII) and cortisone thiolacetate (Δ^4-pregnene-3,11,20-



trione-17α-ol-21-thiol-21-acetate, XIX) were prepared by reaction with potassium thiolacetate in acetone from the corresponding iodides and tosylates. The substances proved to be identical with the substances prepared by Djerassi and Nussbaum [4]. Finally compound S acetate (XX) was obtained from compound S tosylate (IV) by reaction with potassium acetate in acetone.

EXPERIMENTAL

Microanalyses by G. Cornali. All melting points are uncorrected.

Δ^4-pregnene-3,20-dione-17α,21-diol-21-tosylate (IV)

A. *With tosyl chloride and pyridine at low temperature.* A solution of 1.0 g (0.00289 mole) of compound S (I) in 10 ml of dry pyridine was placed in a bath of dry ice and acetone. 600 mg (0.00315 mole) of tosyl chloride (which had been dried by solution in a little benzene and subsequent evaporation of the benzene) was dissolved in 6 ml of dry methylene chloride, and the solution was cooled to such an extent that the tosyl chloride just remained in solution. This solution was poured rapidly into the solidified solution of I. By removing the flask at intervals from the bath and rotating the flask a clear solution was obtained. This solution was allowed to stand in the bath for 2 hours and subsequently at +2° C for 18 hours. The solution was now diluted with ether and washed twice with water, three times with dilute hydrochloric acid, four times with dilute sodium carbonate solution and twice with water. After drying over sodium sulphate the solution was evaporated to dryness. The residue was dissolved in 5 ml of methanol. This solution was filtered and allowed to stand. The crystals which separated were collected and air-dried to yield 1.0 g (69.2 %) with m. p. 159–160° C. (Found: C 66.95; H 7.34; S 6.34. Calc. for $C_{28}H_{36}O_6S$: C 67.17; H 7.25; S 6.40.) In some cases the m. p. was found to be 97–98° C with vesiculation. (Found: C 65.42; H 7.50; S 6.30. Calc. for $C_{28}H_{36}O_6S$, 1 mole CH_3OH: C 65.38; H 7.57; S 6.02.) Following recrystallization from 96 % ethanol the substance with m. p. 159–160° C melted at 160–161° C. (Found: C 65.20; H 7.72; S 5.81. Calc. for $C_{28}H_{36}O_6S$, 1 mole C_2H_5OH: C 65.90; H 7.75; S 5.86.)

B. *With tosyl anhydride and pyridine at low temperature.* Compound S (1 g) was treated as described under A with the exception that a cooled suspension of 1.1 g (0.00337 mole) of tosyl anhydride in 30 ml of dry methylene chloride was added. The solution was allowed to stand for 18 hours at +2° C and subsequently treated as described under A. The yield was 600 mg (41.5 %) of IV.

C. *With tosyl anhydride and pyridine at room temperature.* To a solution of 1 g of compound S in 10 ml of dry pyridine was added a suspension of 1.1 g of tosyl anhydride in dry methylene chloride. The resulting clear solution was allowed to stand at room temperature for 18 hours and subsequently treated as described under A. The yield was 600 mg (41.5 %) of IV.

D. *With tosyl anhydride and pyridine under reflux.* To a solution of 1 g of compound S in 10 ml of dry pyridine was added a suspension of 1.1 g of tosyl anhydride in 30 ml of dry methylene chloride. The solution was refluxed for 2.5 hours and cooled to room temperature, ether was added, and the treatment proceeded as described under A. The yield was 250 mg (17.3 %) of IV.

Δ^4-pregnene-3,11,20-trione-17α,21-diol-21-tosylate (V)

A. *With tosyl chloride and pyridine at low temperature.* Cortisone (II) (1 g; 0.00277 mole) was treated as described in the case of IV A with the exception that the mixture was allowed to stand for 18 hours at a temperature of −18° C. When the ether had been evaporated, the residue was dissolved in 5 ml of benzene and set aside for a short time. The crystals which formed were collected and air-dried to yield 1.2 g (73.0 %). M. p. 108–109° C with vesiculation. (Found: C 68.87; H 6.89; S 5.37. Calc. for $C_{28}H_{34}O_7S$, 1 mole C_6H_6: C 68.89; H 6.80; S 5.41.) A small sample was dissolved in methanol, the solution was filtered and set aside for a short time. The crystals which formed were collected. M. p. 141–143° C (sinters at about 100° C). (Found: C 64.27; H 7.00; S 5.86. Calc. for $C_{28}H_{34}O_7S$, 1 mole CH_3OH: C 63.71; H 7.01; S 5.86.)

In all calculations in which this substance occurs the molecular weight of cortisone tosylate has been taken to be 592.72 corresponding to $C_{28}H_{34}O_7S$, 1 mole C_6H_6.

B. *With tosyl anhydride and pyridine at room temperature.* Cortisone (1 g) was treated as described in the case of IV C. The residue obtained following the removal of the ether was dissolved in benzene, the crystals formed were collected and air-dried to yield 250 mg (15.2 %) of V.

C. *With tosyl chloride and NaOH solution (Schotten-Baumann).* To a solution of 1 g (0.00277 mole) of cortisone in 30 ml of dioxan was added 300 mg (0.00158 mole) of tosyl chloride. 10 ml (0.00158 mole) of aqueous NaOH solution (6.3 g—1 000 ml) was then added dropwise while stirring in the course of 30 min. Another 300 mg of tosyl chloride was now added followed by 10 ml of the abovementioned NaOH solution in the course of 3/4 hour. Ether was added, and the mixture was washed four times with dilute sodium carbonate solution and three times with water. After drying over sodium sulphate and filtration the solution was evaporated to dryness. The residue was dissolved in a little benzene and set aside for a short time. The crystals which formed were collected and air-dried to yield 800 mg (48.6 %) of V.

Δ^4-pregnene-3,20-dione-11β,17α,21-triol-21-tosylate (VI)

Compound F (III) (1 g; 0.00276 mole) was treated as described in the case of V A. The residue obtained after the evaporation of the ether crystallized by the addition of methanol. The solid was collected, washed with methanol and air-dried to yield 800 mg (52.8 %). M. p. 119—121° C with vesiculation. (Found: C 63.37; H 7.20; S 5.67. Calc. for $C_{28}H_{36}O_7S$, 1 mole CH_3OH: C 63.48; H 7.35; S 5.84.)

Δ^4-21-iodopregnene-3,20-dione-17α-ol (VII)

To a solution of 1 g (0.0020 mole) of IV in 5 ml of acetone was added a solution of 1 g (0.0067 mole) of sodium iodide in 7 ml of acetone. In the course of a few minutes separation of sodium tosylate started. The mixture was refluxed for 7—8 minutes, most of the acetone was distilled off, and the solution was cooled to room temperature. On the addition of dilute sodium thiosulphate solution and water a white precipitate was formed. This precipitate was collected, washed thoroughly with water and air-dried to yield 870 mg (95.4 %) of analytically pure VII. M. p. 135—136° C dec. (the substance turns dark at about 125° C). (Found: C 55.06; H 6.59; I 27.70. Calc. for $C_{21}H_{29}IO_3$: C 55.26; H 6.41; I 27.81.)

Δ^4-21-iodopregnene-3,11,20-trione-17α-ol (VIII)

Cortisone tosylate (V) (1 g; 0.0017 mole) was treated as described in the case of VII. The substance obtained was dried to yield 780 mg (98.3 %) of VIII, decomposition of which starts at about 140° C. Following recrystallization from dioxan of a small sample, it was found to have m. p. 155—156° C dec. (Found: C 53.71; H 5.99; I 26.79. Calc. for $C_{21}H_{27}IO_4$: C 53.62; H 5.79; I 26.98.)

Δ^4-21-iodopregnene-3,20-dione-11β,17α-diol (IX)

Compound F tosylate (VI) (1 g; 0.0018 mole) was treated as described in the case of VII. The substance obtained was dried to yield 750 mg (87.1 %) of IX. M. p. 148—149° C dec. (Found: C 53.57; H 6.30; I 26.60. Calc. for $C_{21}H_{29}IO_4$: C 53.39; H 6.19; I 26.87.)

Δ^4-21-bromopregnene-3,20-dione-17α-ol (X)

A solution of 0.5 g (0.0010 mole) of IV in 5 ml of dry methanol was prepared by heating on a steam bath, and a solution of 350 mg (0.0034 mole) of NaBr in 10 ml of dry methanol was added. The resulting solution was refluxed for 3 hours. Half of the methanol added was distilled off and the residue cooled in running water. The precipitate which formed was collected, washed thoroughly with water and dried to yield 300 mg (73.4 %) of X. M. p. 223—224° C dec. (Found: C 61.73; H 7.25; Br 19.55. Calc. for $C_{21}H_{29}BrO_3$: C 61.61; H 7.14; Br 19.52.)

Δ^4-21-bromopregnene-3,11,20-trione-17α-ol (XI)

Cortisone tosylate (V) (0.5 g; 0.0008 mole) was treated as described in the case of X. The 21-bromo compound started to crystallize during the boiling. After refluxing for three hours and cooling in running water, the solid was collected, washed thoroughly with water and dried to yield 300 mg (85.0 %) of XI with m. p. 249—250° dec.*. (Found: C 59.54; H 6.58; Br 18.75. Calc. for $C_{21}H_{27}BrO_4$: C 59.57; H 6.43; Br 18.88.)

Δ^4-21-chloropregnene-3,20-dione-17α-ol (XII)

To a solution of 0.5 g (0.0010 mole) of IV in 25 ml of dry ethanol was added a filtered solution of 1 g (0.0090 mole) of dried $CaCl_2$ in 25 ml of dry ethanol. The solution was refluxed for 5 hours. Most of the ethanol was distilled off and water was added. The precipitate was collected, washed with water and dried to yield 350 mg (96.0 %) of XII. M. p. 234—235° C dec. (Found: C 68.90; H 7.88; Cl 9.59. Calc. for $C_{21}H_{29}ClO_3$: C 69.12; H 8.01; Cl 9.72.)

Δ^4-21-chloropregnene-3,11,20-trione-17α-ol (XIII)

Cortisone tosylate (V) (0.5 g; 0.0008 mole) was treated as described in the case of XII. The precipitate was collected, washed with water and air-dried to yield 300 mg (93.9 %) of analytically pure XIII. M. p. 282—284° C dec.**. (Found: C 66.48; H 7.45; Cl 9.40. Calc. for $C_{21}H_{27}ClO_4$: C 66.57; H 7.18; Cl 9.36.)

To a solution of 0.5 g (0.0093 mole) of NH_4Cl in 150 ml of dry ethanol prepared by heating was added 0.5 g of V and the solution was refluxed for 5 hours. Most of the ethanol was distilled off and water was added. The precipitate was collected, washed with water, dried and recrystallized from 99 % C_2H_5OH to yield 275 mg (86.0 %) of XIII. M. p. 282—284° C dec. Identity with a sample prepared with $CaCl_2$ was established by a mixed melting point.

Δ^4-21-cyanopregnene-3,20-dione-17α-ol (XIV)

To a solution of 0.5 g (0.0011 mole) of VII in 70 ml of methanol was added 0.5 g (0.0077 mole) of KCN. The solution was refluxed for 10 min. and most of the methanol was then distilled off in vacuum. Water was added and the precipitate collected, washed with water and dried to yield 300 mg of a crude product, m. p. 210—225° C. After recrystallization from methanol the yield was 150 mg (38.5 %), m. p. 249—251° C. (Found: C 74.13; H 8.45; N 3.90. Calc. for $C_{22}H_{29}NO_3$: C 74.33; H 8.22; N 3.94.)

Δ^4-21-cyanopregnene-3,11,20-trione-17α-ol (XV)

To a solution of 0.5 g (0.0011 mole) of VIII in 80 ml of methanol was added 200 mg (0.0031 mole) of KCN dissolved in 4 ml of water. The solution was refluxed for 10 min. Most of the methanol was then distilled off in vacuum and a solution of sodium chloride was added. The precipitate was collected, washed with water and dried to yield 175 mg of a crude product with m. p. 210—220° C. Recrystallization from 96 % C_2H_5OH yielded 75 mg (19.1 %) with m. p. 236—237° C. (Found: C 71.53; H 7.63; N 3.82. Calc. for $C_{22}H_{27}NO_4$: C 71.52; H 7.37; N 3.79.)

Δ^4-21-thiocyanopregnene-3,20-dione-17α-ol (XVI)

To a solution of 0.5 g (0.0011 mole) of VII in 40 ml of dry acetone was added 0.5 g (0.0052 mole) of dried KSCN (benzene was poured over KSCN and then distilled off). The solution was refluxed for 3.5 hours and most of the acetone subsequently distilled off. Water was added, and the precipitate collected, washed with water and dried to yield 400 mg (94.2 %) of XVI with m. p. 188—189° C. Recrystallization from methanol did not raise the melting point. (Found: C 68.14; H 8.00; S 8.17; N 3.55. Calc. for $C_{22}H_{29}NO_3S$: C 68.18; H 7.54; S 8.27; N 3.62.)

* Velluz, L., Warnandt, J., Nominé, G., Joly, R. and Petit, A. *(Bull. soc. chim. France* (5) **20** (1953) 906) found m. p. 297—300° C.

** J. Leanza and coworkers [5] found m. p. 243—245° C dec.

Δ^4-21-thiocyanopregnene-3,11,20-trione-17α-ol (XVII)

The 21-iodo compound (VIII) (0.5 g; 0.0011 mole) was treated as described in the case of XVI. The yield obtained was 420 mg (98.4 %) of XVII with m. p. 193—194° C. Recrystallization from benzene did not raise the melting point. (Found: C 66.05; H 6.86; N 3.46; S 7.98. Calc. for $C_{23}H_{27}NO_4S$: C 65.81; H 6.78; N 3.49; S 7.98.)

To a solution of 0.5 g (0.0008 mole) of V in 40 ml of dry acetone was added 0.5 g of dried KSCN. The solution was refluxed for 3.5 hours and most of the acetone was subsequently distilled off. Water was added and the precipitate collected, washed with water and dried to yield 300 mg (88.6 %) of XVII with m. p. 192—193° C. The melting point was not depressed on admixture with the substance prepared from VIII.

Δ^4-pregnene-3,20-dione-17α-ol-21-thiol-21-acetate (XVIII)

To a solution of 1 g (0.0022 mole) of VII in 50 ml of dry acetone was added 650 mg (0.0057 mole) of potassium thiolacetate, and the mixture was refluxed for 2.5 hours and most of the acetone was subsequently distilled off. Water was added and the precipitate collected, washed with water and dried to yield 750 mg (84.6 %) of XVIII with m. p. 214—217° C. Recrystallization from 99% ethanol raised the m. p. to 219—222° C. (Found: C 68.22; H 7.88; S 7.72. Calc. for $C_{23}H_{32}O_4S$: C 68.28; H 7.97; S 7.93.)

Δ^4-pregnene-3,11,20-trione-17α-ol-21-thiol-21-acetate (XIX)

To a solution of 1 g (0.0021 mole) of VIII in 50 ml of dry acetone was added 650 mg of potassium thiolacetate, and the mixture was refluxed for 2.5 hours. Most of the acetone was then distilled off, and after addition of water the precipitate was collected, washed with water and dried to yield 650 mg (73.0 %) analytically pure XIX with m. p. 224—226° C. Recrystallization from methanol did not raise the melting point. (Found: C 65.83; H 7.30; S 7.36. Calc. for $C_{23}H_{30}O_5S$: C 66.00; H 7.23; S 7.66.)

To a solution of 1 g (0.0017 mole) of V in 50 ml of dry acetone was added 600 mg of potassium thiolacetate, and the mixture was refluxed for 2.5 hours. Most of the acetone was then distilled off, and after addition of water the precipitate was collected, washed with water and dried to yield 500 mg (70.8 %) of XIX with m. p. 224—226° C. Identity with a sample prepared from VIII was established by a mixed melting point.

Δ^4-pregnene-3,20-dione-17α,21-diol-21-acetate (XX)

To a solution of 0.5 g (0.0010 mole) of IV in 50 ml of dry acetone was added 250 mg (0.0025 mole) fused potassium acetate, and the mixture was refluxed for 4.5 hours. Most of the acetone was then distilled off, water was added and the precipitate was collected, washed with water and air-dried to yield 300 mg (77.3 %) of XX with m. p. 228—231° C. Identity with a sample of compound S acetate was established by a mixed melting point.

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On the Alkaline Error of the Glass Electrode *

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A theoretical treatment is given to the glass electrode alkaline behaviour, considering the influence of the state of adsorption on the adsorption energies. By expanding in series and approximating, the equation deduced has been transformed into earlier known, empirical expressions. Experimental results confirm the theoretical treatment. Finally a differential measuring method has been described, to be applied for correcting the alkaline error in glass electrode measurements when the activity of the sodium ion (or other) is unknown, comprising the use of two glass electrodes of different, but known, alkaline error functions.

Since Hughes [1] discovered the alkaline error of glass electrodes in 1922, several theories have been applied to the quantitative treatment of the difference between hydrogen and glass electrode behaviour. Important contributions in this field have been made by Dole *et al.*[2–7], and also by Nicolsky [8], Gross and Halpern [9] and Tendeloo and Voorspuij [10, 11]. For a more general review of the different theories, reference may be made to Kratz [12].

The theories of Dole and of Nicolsky give the same final equation, which can be written:

$$\log[\exp\ (F\triangle E/RT) - 1] = \mathrm{pH} + \log\ C_{Na+} + \text{constant} \qquad (1)$$

($\triangle E$ is the alkaline error, pH and C_{Na+} refer to the solution surrounding the glass electrode.) If log [exp $(F\triangle E/RT) - 1$] is plotted as a function of pH from experimental data, the linear agreement is good, except that the slopes of the lines are not unity, as demanded by eq. (1). Thus, for an electrode made of Corning 015 glass, a slope of 0.57 for 1 M Na^+ at 25° C was found by Dole [13]. (It was also stated in the same paper that no quantitative explanation of the deviation of the slope from unity had been advanced at that time.)

The validity of the Gross and Halpern treatment has been discussed and criticised by Dole [7], who concludes that their final equation is not as good as eq. (1) for explaining glass electrode behaviour in alkaline solutions.

* This paper is based on a lecture given by the author at the 8:th Congress of Scandinavian Chemists in Oslo, June 14—17, 1953.

The treatment by Tendeloo and Voorspuij gives the equation:

$$\log [\exp (F\triangle E/RT)-1] = \mathrm{pH} + \log [(1 + K_2' \cdot C_{Na+})/K'_1] \quad (2)$$

($\triangle E$ is the alkaline error, pH and C_{Na+} refer to the surrounding solution, K_1' and K_2' are electrode constants.) The equation is very similar to eq. (1), and for ordinary glass electrodes it fails as does eq. (1) in predicting a slope of unity. However, these authors used an electrode made of Al_2O_3-containing glass with a very high alkaline error. At pH 11 and $C_{Na+} = 0.1$ the error was about 50 times greater than that of an electrode made of Corning 015 glass. In this case eq. (2) is in good agreement with experimental results, but the lack of agreement occurs at low $\triangle E$, which can be expected from the behaviour of said Corning 015 glass. This is also stated by the authors.

The basic ideas of the four different treatments refered to above, have been:

Dole: Ion exchange reactions take place between the glass electrode surface and the surrounding solution, and the equilibrium is treated mathematically by a statistical method used earlier by Gurney [14,15] in studies on other electrode reactions.

Nicolsky: The ion distribution on the glass surface and in the surrounding solution is treated by the law of mass action.

Gross and Halpern: "It is assumed that the electrode glass may be treated as a difficulty soluble salt, which forms with water a saturated solution, so that the water dissolves in the glass until the glass becomes saturated with water. It is also assumed that the mass action law holds for such strong electrolytes as sodium hydroxide and sodium acetate both in water and glass phases, that water, glass, acids, bases *etc.* distribute themselves between the glass and the aqueous phase in accordance with the well-known distribution law, and that the activity coefficients of all ions are the same." (Dole [7]).

Tendeloo and Voorspuij: Dissociation equilibria between the swollen glass surface and the surrounding solution are controlled by real constants of dissociation for the silicic acid in that surface.

In the following discussion a refined treatment of the glass electrode alkaline error will be given, and the influence of the state of adsorption on the adsorption energies will also be considered. This has not been made in the treatments mentioned above.

The *physical model* used for the mathematical treatment is in accordance with the ideas of Tendeloo and Voorspuij [11], the SiO_4-network of the electrode glass being regarded as containing three dimentional interstices, offering room for metal ions such as Na^+ and Ca^{2+}. When the electrode surface is brought in contact with water the network on the surface and to some depth into the glass will pass into a swollen state, the aforesaid metal ions will be dissolved to a great extent and replaced by H^+ or H_3O^+ ions. In this way the *surface layer* is substantially free from other cations, and consists of silicic acid. The silicic acid is then able to take part in ion exchange reactions, so that all states from complete H^+ saturation to a more or less mixed composition of H^+ and other cations are possible. Under these conditions it may be anticipated that the adsorption energies, *i.e.* the forces between adsorbed ions and the adsorption sites, will be dependent on the state of adsorption, so that the energy will not be the same if an adsorbed ion has identical or dissimilar ions as neighbours

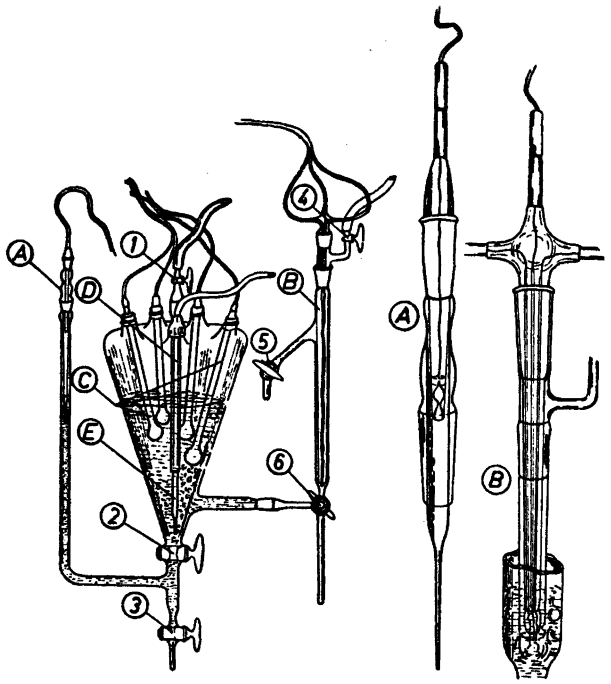


Fig. 1. Apparatus for hydrogen and glass electrode measurements.

on the surrounding adsorption sites. This seems to be reasonable, as the degree of "energy saturation" of each adsorption site may influence the surrounding sites, with regared to their available adsorption forces. As we are dealing with a network, *i.e.* a kind of a large molecule, the state of the internal resonance energies can to some extent be compared with that of a polybasic acid. However, in the case of glass the effect is more complex, as the glass is a solid acid, where the electrical forces outside the molecule have a large influence on the activity of the adsorbed ions.

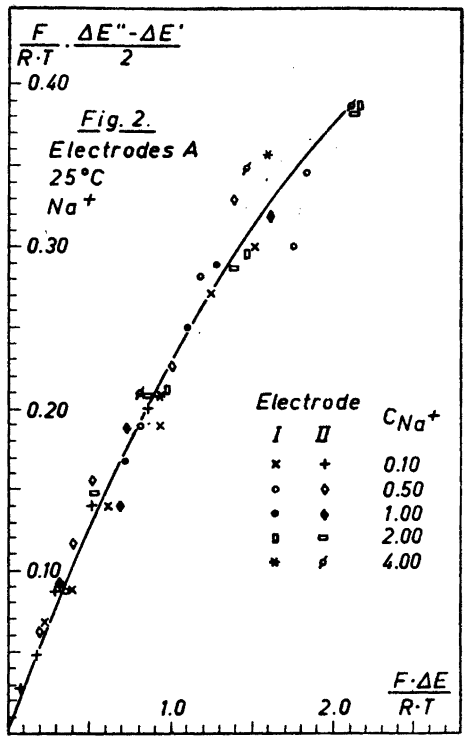
EXPERIMENTAL

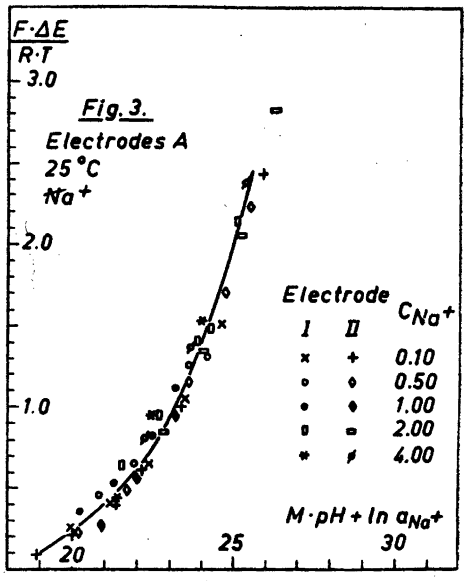
In the experiments a glass apparatus, Fig. 1, was used, with spaces for four glass electrodes C, a reference electrode A, a hydrogen electrode B and a temperature compensator D, used when measurements were made with instruments having an automatic temperature compensation device. During the measurements the whole system was enclosed in a Faraday cage, and the temperature of the surrounding room, 25° C, controlled by means of a radiant heating device to within 0.1° C, with a temperature change less than 0.1° C per hour. When hydrogen electrodes are used in alkaline solutions, it is of greatest importance that the hydrogen should be of a very high purity. An amount of oxygen exceeding 300 μg per litre can give mixed hydrogen-hydrogen ion and oxygen-hydroxyl ion potentials, disturbing the response of the electrode. The cylinder hydrogen used in this work was purified in the following manner, which gave excellent results. The gas was passed through a tube of stainless steel, with a diameter of 20 millimeters, con-

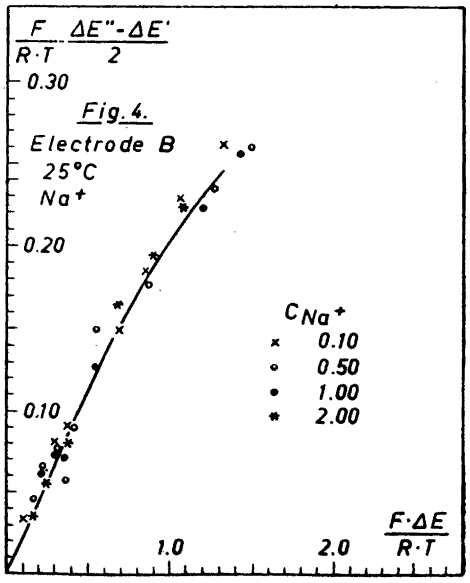
taining one layer of cut copper wires, and another of platinum deposited on porcelain. The height of each layer was 300 millimeters. The tube was heated by a 1 000 watt heating coil, controlled by an energy regulating device to maintain the temperature of the catalyst at 650 ± 25° C. The gas was in contact with the catalyst for about 5 minutes. It was then passed through a copper pipe for air cooling and bubbled through concentrated sulphuric acid and then through a solution of sodium hydroxide (400 g per litre). Before entering the hydrogen electrode, the gas was passed through a large quantity of thermostated distilled water, in order to avoid temperature changes at the electrode and to attain approximate equilibrium with the vapor pressure of the solution to be investigated. This eliminated changes in concentration, caused by evaporation from the electrode solution. The efficiency of the purification was examined from time to time. The purified hydrogen was bubbled through a small volume of 0.1 *M* KCl, and after one hour of bubbling the oxygen was determined polarographically. The amount of oxygen was always found to be less than a few μg per litre, which was below the limit for side reactions. Anodic polarographic investigations in 0.1 *M* Na_2HPO_4 gave no detectable wave of H_2S, arising from the sulphuric acid and not completely removed by the sodium hydroxide solution. From this it can be concluded that the amount of hydrogen sulphide was less than a few μg per litre of solution.

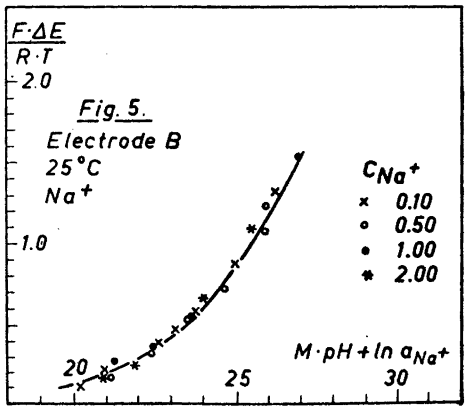
The hydrogen electrode B was designed as a triple electrode with three separate platinum wires for measuring the response of the electrode. In every pH measurement the three electrodes were measured individually several times, and the agreement of the three potentials had to be within the accuracy of the instruments used for the determination, before the measurements were accepted. Experience has shown that oxygen impurities, bad platinum plating and other electrode errors do not give identical potentials. The platinum plating was prepared by electrolysis, using a Pt anode, area 200 mm^2, at a distance from the cathodes of 20—30 mm, in 20 % $PtCl_4$ at 4 volts for 15 sec. and at a temperature of 20° C. The cathodes, *i. e.* the three platinum wires of the hydrogen electrode, were each 0.5 mm in diameter and 7 mm in length. The platinum deposit was renewed when necessary, but at least every day. The old deposit was carefully removed and the electrode cleaned by means of sodium chromate in sulphuric acid and rinsed in distilled water before replating. The reference electrode was a 0.1 *M* calomel electrode, this concentration being used as it has a low temperature coefficient and seems, with the electrode design used here to be practically free from variations in diffusion potentials. The electrode was similar to the type introduced by Perley [16].

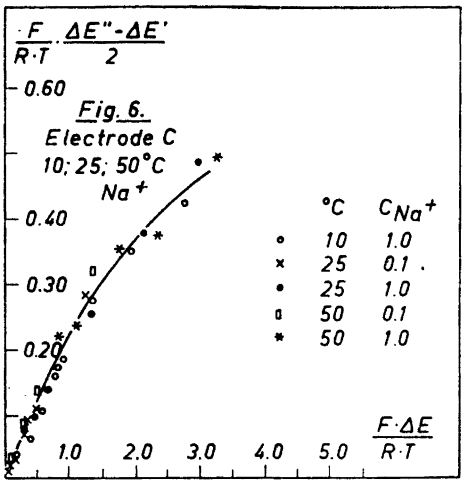
The apparatus was operated in the following manner: With the cocks 2 and 6 (without grease) and 5 open, the test solution, buffer I or II, was introduced into the apparatus to cover the electrodes. (The reference electrode has a little hole, not shown, to allow the air to escape.) After closing the cocks 2 and 6 and opening 1 and 4, the hydrogen bubbling was started. As the hydrogen electrode space has a small volume, the oxygen in the solution was removed and hydrogen saturation quickly obtained. The minimum time of bubbling was of the order 10—15 minutes. The glass electrode potentials were measured at 5 minute intervals over a period of 30 minutes, thus giving 6 different determinations on each solution. The solution around the glass electrodes was stirred continously by means of the bubbling hydrogen, introduced by means of the pipe E. After 30 minutes the hydrogen electrode potential was determined, and during this measurement, cock 5 was closed, and all the three electrodes tested for coincidence. In order to investigate if any liquid junction potentials arise at the cocks 2 and 6, these cocks were opened and the potential difference determined. To change the pH of the solution, the cocks 1 and 5 were opened and the hydrogen electrode tube and that containing the reference electrode drained by means of cocks 2, 3 and 6. A desired amount of "contrabuffer" II or I was added to the solution around the glass electrodes, which was then stirred violently by means of the hydrogen, and by using the cocks 2, 3 and 6 the hydrogen and reference electrodes were rinsed several times, and finally as much solution removed as was necessary to maintain constant liquid level. The next measurements could then be made. Before and after each series of measurements the apparatus was filled with a control buffer and hydrogen and reference electrodes and asymmetry potential changes checked. As a rule identical values were found, but if this was not the case, the whole series of measurements were rejected and repeated after careful checking of the electrode system. The average accuracy was ± 0.25 mV.

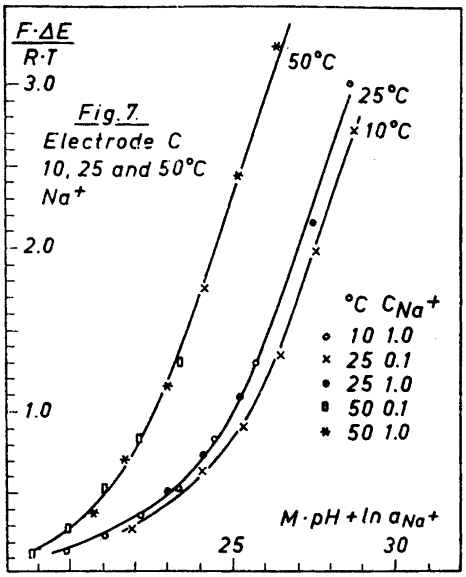

F/R·T · (ΔE''-ΔE')/2
0.40
Fig. 2.
Electrodes A
25 °C
Na+
0.30
0.20
0.10
Electrode
I II
C Na+
0.10
0.50
1.00
2.00
4.00
1.0
2.0
F·ΔE/R·T


F·ΔE/R·T
3.0
Fig. 3.
Electrodes A
25 °C
Na+
2.0
1.0
Electrode
I II
C Na+
0.10
0.50
1.00
2.00
4.00
20
25
30
M·pH + ln a Na+


F/R·T · (ΔE''-ΔE')/2
0.30
Fig. 4.
Electrode B
25 °C
Na+
0.20
0.10
C Na+
0.10
0.50
1.00
2.00
1.0
2.0
F·ΔE/R·T

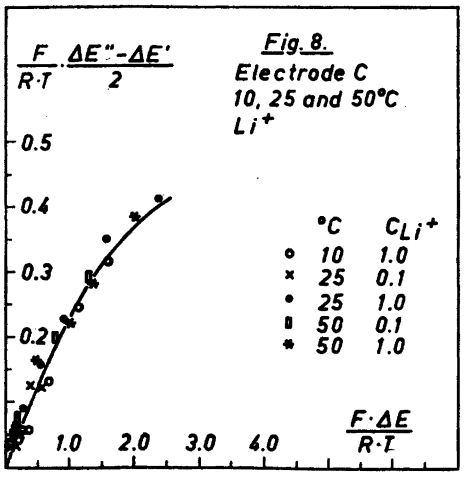

F·ΔE/R·T
2.0
Fig. 5.
Electrode B
25 °C
Na+
1.0
C Na+
0.10
0.50
1.00
2.00
20
25
30
M·pH + ln a Na+

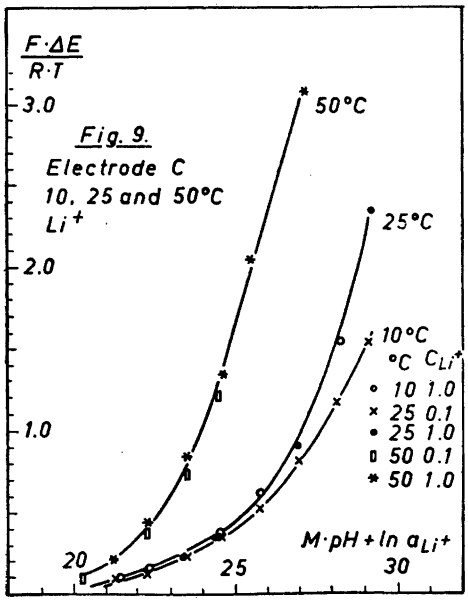


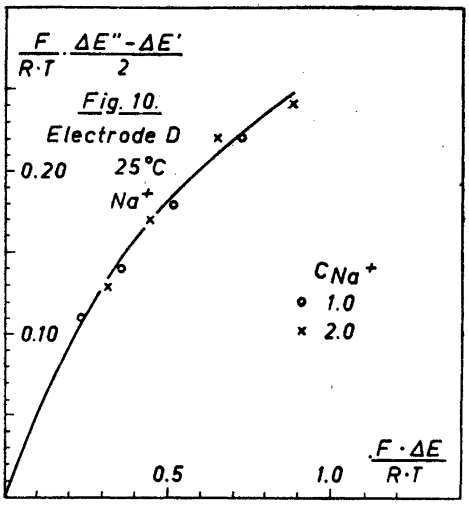


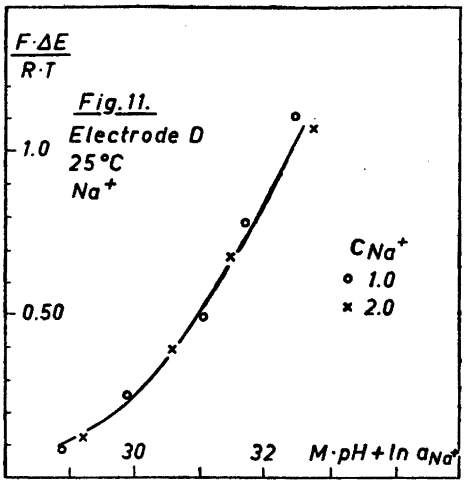
The buffer solutions used were: I. 0.010 *M* Na_3PO_4 + 0.010 *M* Na_2CO_3 + 0.050 *M* NaOH + NaCl to C_{Na+} = 0.10, 0.50, 1.00, 2.00 and 4.00. II. The same as I, but with 10 ml per litre 10 *M* HCl added. Control buffer: 0.025 *M* KH_2PO_4 + 0.025 *M* Na_2HPO_4; pH 6.86.

The instruments used for the potential determinations were a Radiometer PHM 3g and two different Leeds & Northrup instruments as secondary controls.

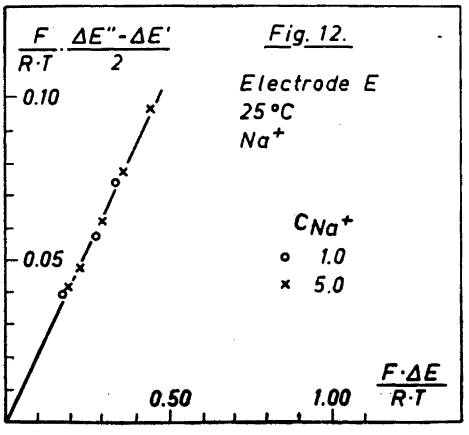


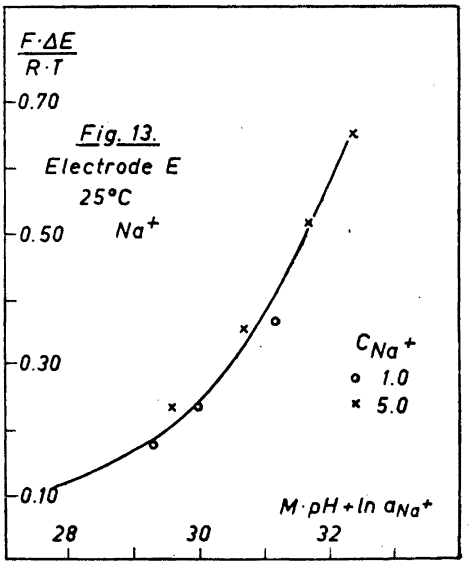






Measurements were made on two commercial glass electrodes, one of type A, and one of another type B, using the method described above. The investigated ranges were: pH 9—11.5 and C_{Na+} 0.10—4.00 at a temperature of 25° C. The electrodes A and B have different, but unknown glass composition. For calculations in accordance with the theory presented later in this paper, data obtained by Dole *et al.*[2-7, 12] have also been used. The object of this has been to include still another composition of glass, and also to include the effect of different temperatures, 10, 25 and 50° C, and of different ions, Na+ and Li+. (As the other ions investigated by Dole give a very low alkaline error, the accuracy is not sufficient for these calculations. Hence only the Na+ and Li+ results have been examined.)





In the present paper, the electrode used by Dole has been denoted by C. Figures for Na^+ at 25° C from the manufacturers' bulletins concerning two different electrodes D and E, having a very low alkaline error, were also used as a basis for mathematical calculations. In this case the data are of doubtful value, as nothing is known of the measuring technique and accuracy, the test solution composition and the activity changes at high pH values. The assumption has been made that NaCl was present in the solution and that the amount of buffer was low. The values have been included since they do give some additional indication of the low alkaline error electrode behaviour.

For the electrodes mentioned above, two functions have been plotted for all of them from experimental data, to be used in the theoretical treatment, namely:

$$F(\triangle E'' - \triangle E')/2\ RT = f(F\triangle E/RT) \tag{3}$$

$$F\triangle E/RT = g(\mathrm{M.pH} + \ln a_{Na+}) \tag{4}$$

In these functions $\triangle E$ is the alkaline error at an activity of hydrogen ions corresponding to pH and a sodium activity of a_{Na+}. $\triangle E''$ is the alkaline error at a pH 0.25 units above the pH value at $\triangle E$, and $\triangle E'$ is that at a pH 0.25 below. M is the logarithm conversion figure. In the case of eq. (4) the sodium ion activity has to be estimated. This is, of cause, a difficult task. In the measurements made by the author, the buffer concentration was kept low and most of the sodium ions are due to NaCl. This gives a simpler solution from the point of view of activity calculations. In all cases the mean activity coefficient of the electrolyte has been applied to Na^+, and has been taken from different papers [17-19]. However, even if the accuracy of the activity figures is moderate, they are to be preferred to the concentration figures used in other treatments. In Figs. 2—13 plots of the functions (3) and (4) are shown, and the mean curve from them will be used later for further calculations.

THEORETICAL

Applied to a system where ions are distributed between different phases, the Boltzmann energy distribution law can be written:

$$_iC_j = \exp(-{}_i\varepsilon_j/RT) \tag{5}$$

where $_iC_j$ = concentration of an ion i in a phase j; $_i\varepsilon_j$ = total energy for an ion i in a phase j. If considering the physical model, and eq. (5) is applied to one of the two glass electrode surfaces, *i.e.* to the system solution — surface layer, then:

$$_iC_s = \exp(-{}_i\varepsilon_s/RT) \tag{5a}$$

$$_iC_g = \exp(-{}_i\varepsilon_g/RT) \tag{5b}$$

$$_iC_g = {}_iC_s \cdot \exp[-({}_i\varepsilon_g - {}_i\varepsilon_s)/RT] \tag{6}$$

where: $_iC_s$ = concentration in the solution of an ion i, $_iC_g$ = concentration of an ion i in the *surface layer* of the glass, defined in accordance with the *physical model*, $_i\varepsilon_s$ = total energy in the solution for an ion i, $_i\varepsilon_g$ = total energy in the surface layer for an ion i.

Let: $_iU_s$ = equilibrium energy in the solution for an ion i, $_iU_g$ = equilibrium energy in the surface layer for an ion i.

Then:

$$_i\varepsilon_s = {}_iU_s + \text{constant}$$

$$_i\varepsilon_g = {}_iU_g + zFE + \text{constant}$$

where E is the difference in electrical potential between solution and surface layer, and z is the valency of i.

From this:

$$ {}_iC_g = {}_iC_s \cdot \exp\left[-({}_iU_g + zFE - {}_iU_s)/RT\right] \quad (6b) $$

As the logarithm of the activity coefficient gives the difference between actual and ideal energies in the solution, then if the ideal energy is written ${}_iU_{os}$:

$$ {}_iC_g = {}_ia_s \cdot \exp\left[-({}_iU_g + zFE - {}_iU_{os})/RT\right] \quad (7) $$

where ${}_ia_s$ is the activity of the ion in the solution.

If we define: ${}_iU_{os} - {}_iU_g = Q_i$
then

$$ \Sigma\, {}_ia_s \cdot \exp\left[(Q_i - zFE)/RT\right] = \Sigma\, {}_iC_g \quad (8) $$

This can be regarded as the fundamental equation of the glass electrode.

In the following treatment we shall for simplicity select the ions H_3O^+ and Na^+. In this case eq. (8) can be written:

$$ E = \frac{RT}{F} \ln\left[a_{H_3O+} \cdot \exp\left(Q_{H_3O+}/RT\right) + a_{Na+} \cdot \exp\left(Q_{Na+}/RT\right)\right] - \frac{RT}{F} \ln \Sigma\, {}_iC_g \quad (9) $$

Let $X_i = {}_iC_g/\Sigma_i C_g$, and define $Q_i \to Q_{oi}$ ${}_iC_g \to {}_iC_{og}$ when $X_i \to 1$.
If $a_{Na+} \cdot \exp\left(Q_{Na+}/RT\right) \ll a_{H_3O+} \cdot \exp\left(Q_{H_3O+}/RT\right)$ eq. (9) can be written:

$$ E' = Q_{oH_3O+}/F - \frac{RT}{F} \ln \Sigma\, {}_iC_{og} - \frac{MRT}{F}\,\mathrm{pH} \quad (10) $$

Let

$$ Q_{oH_3O+}/F - \frac{RT}{F} \ln \Sigma\, {}_iC_{og} = e_o $$

then

$$ E' = e_o - \frac{MRT}{F}\,\mathrm{pH} \quad (10a) $$

This is, except e_o, the equation for the hydrogen electrode. The differences in e_o for the two active surfaces of the glass electrode:

$$ e_o'' - e_o' = \varphi \quad (11) $$

is an expression of the *asymmetry potential, i.e.* the deviation in E_o in comparison with the hydrogen electrode. If the electrode surfaces change their properties in different ways, this will cause a variation in φ. However, for short periods (of the order 100 hours) φ is a constant.

If it is not possible to neglect the Na^+-term, and if we define:

$$ \Delta E = E - E' \quad (12) $$

then, since

$$ \Sigma\, {}_iC_g/\Sigma\, {}_iC_{og} = X_{H_3O+} + X_{Na+} $$

$$ (X_{H_3O+} + X_{Na+}) \cdot \exp\left[(Q_{oH_3O+} - Q_{H_3O+})/RT\right] \cdot \exp\left(F\Delta F/RT\right) = $$

$$ \frac{a_{Na+}}{a_{H_3O+}} \exp\left[(Q_{Na+} - Q_{H_3O+})/RT\right] + 1 \quad (13) $$

Applying the distribution law once more:

$$\frac{X_{Na+}}{X_{H_3O+}} = \frac{a_{Na+}}{a_{H_3O+}} \exp\ [(Q_{Na+} - Q_{H_3O+})/RT]$$

and $$\frac{a_{Na+}}{a_{H_3O+}} \exp\ [(Q_{Na+} - Q_{H_3O+})/RT] + 1 = \frac{X_{Na+} + X_{H_3O+}}{X_{H_3O+}}$$

Combining this with eq. (13) gives:

$$F \cdot \Delta E/RT = (Q_{H_3O+} - Q_{oH_3O+})/RT - \ln\ X_{H_3O+} \qquad (14)$$

So far nothing has been assumed concerning the *number* of points for cationic adsorption in the surface layer. Here the assumption will be made that *the number of points is constant.* The reason for this is that only a destruction of the surface layer will change that number, and the possibility of such a destruction seems to be remote. This can be concluded from the fact that the asymmetry potential remains constant, even if the electrode is alternately brought in contact with acid and alkaline solutions, and also from the reproducible alkaline behaviour.

From this assumption we get:

$$\Sigma_i C_g = \Sigma_i C_{og} = \text{constant} \qquad (15)$$

and $$X_{H_3O+} + X_{Na+} = 1 \qquad (16)$$

It may be pointed out that eq. (16) contains *concentrations, i.e. numbers* of ions. However, the *activity* of the adsorbed ions may change with the state of adsorption, and this will be revealed by variations in U_g and Q_i.
By applying eq. (16), eq. (13) is reduced to:

$$\exp\,[(Q_{oH_3O+} - Q_{H_3O+})/RT] \cdot \exp\,(F\Delta E/RT) = \frac{a_{Na+}}{a_{H_3O+}} \exp[(Q_{Na+} - Q_{H_3O+})/RT] + 1 \qquad (17)$$

From the treatment given above it is possible to calculate X_{H_3O+}, X_{Na+}, $(Q_{H_3O+} - Q_{oH_3O+})/RT$ and $(Q_{oH_3O+} - Q_{Na+})/RT$ from experimental data on the alkaline error under different conditions. This can be done from two separate values of both ΔE and a_{Na+}/a_{H_3O+} by means of eq. (14) and eq. (17). The first step is to determine $\exp\,[(Q_{oH_3O+} - Q_{H_3O+})/RT]$, keeping a_{Na+} constant.

In practice it is not possible to attain these conditions, as the sodium ion activity changes with the ionic strength when pH is altered. However, errors from this source can be kept low if a suitable solution is used, containing a small amount of buffer with most of the sodium ions present as NaCl. We write:

$$\exp\ [(Q_{oH_3O+} - Q_{H_3O+})/RT] = \gamma$$

$$\exp\ [(Q_{Na+} - Q_{H_3O+})/RT] = \delta$$

From eq. (17) $$\gamma \cdot \exp\ (F\Delta E/RT) = \delta \frac{a_{Na+}}{a_{H_3O+}} + 1$$

and for $\triangle E''$ and $\triangle E'$

$$\gamma \cdot \exp\ (F\triangle E''/RT) = \delta \frac{a_{Na+}}{a''_{H_3O+}} + 1$$

$$\gamma \cdot \exp\ (F\triangle E'/RT) = \delta \frac{a_{Na+}}{a'_{H_3O+}} + 1$$

i.e.

$$\frac{\gamma \cdot \exp\ (F\triangle E'/RT) - 1}{\gamma \cdot \exp\ (F\triangle E''/RT) - 1} = \frac{a''_{H_3O+}}{a'_{H_3O+}}$$

($\triangle E' < \triangle E''$ and correspondingly $a'_{H_3O+} > a''_{H_3O+}$)

With a pH difference of 0.50, which is adequate for over all accuracy in the measurements, we have: $a''_{H_3O+}/a'_{H_3O+} = 1/\sqrt{10}$ and

$$\frac{1}{\gamma} = \exp\left[(Q_{H_3O+} - Q_{oH_3O+})/RT\right] = \frac{3.16\ \exp(F\triangle E'/RT) - \exp\ (F\triangle E''/RT)}{2.16} \quad (18)$$

Putting as a first approximation $\triangle E = (\triangle E' + \triangle E'')/2$, which is equivalent to assuming that the alkaline error function is a straight line in the small interval between $\triangle E'$ and $\triangle E''$, and combining eq. (14) and eq. (18):

$$X_{H_3O+} = \frac{1.46}{\exp\ (F(\triangle E'' - \triangle E')/2RT]} - 0.462 \cdot \exp\ [F(\triangle E'' - \triangle E')/2RT] \quad (19)$$

From the earlier equation:

$$\frac{X_{Na+}}{X_{H_3O+}} = \frac{a_{Na+}}{a_{H_3O+}} \exp\ [(Q_{Na+} - Q_{H_3O+})/RT]$$

and eq. (14) we get:

$$\ln\ X_{Na+} + (Q_{oH_3O+} - Q_{Na+})/RT = \ln a_{Na+} + M \cdot pH - F\triangle E/RT \quad (20)$$

As $X_{H_3O+} + X_{Na+} = 1$ we can derive X_{Na+} and $(Q_{oH_3O+} - Q_{Na+})/RT$ from eq. (19) and eq. (20). As can be seen from above, it is convenient to plot the functions (3) and (4) from the experimental data, as this considerably reduces the calculation required.

In order to deduce an alkaline error equation, it is necessary to find the dependence of $(Q_i - Q_{oi})/RT$ on X_i. We will here start with the assumption earlier used for localized adsorption layers, *e.g.* by Fowler [20], that the energy of interaction can be expressed as the sum of contributions of pairs of nearest neighbours. If every adsorption point has n neighbours, and an ideal distribution is assumed, every site has a possibility X_i of being occupied by an ion i; the number of pairs will be $\frac{n}{2} \cdot X_i$. As it is assumed that the change in interaction energy, for the whole system given as $X_i \cdot d_i U_g$, is proportional to the change in number of pairs, we get:

$$X_i \cdot d_i U_g = k_2 \cdot \frac{n}{2} dX_i \quad (21)$$

Or, if we write $\frac{2}{n k_2} = k_3$,

$$\frac{dX_i}{X_i} = k_3 \cdot d_i U_g \tag{22}$$

and $$\ln X_i = k_3 \cdot d_i U_g + \text{constant} \tag{23}$$

As we defined ${}_iU_g \rightarrow {}_iU_{og}$ when $X_i \rightarrow 1$, the value of the constant is $-{}_iU_{og}$.

and $$\ln X_i = k_3({}_iU_g - {}_iU_{og}) \tag{23a}$$

Regarding the significance of Q_i and Q_{oi} and writing $k_3 \cdot RT = k_4$:

$$\ln X_i = k_4(Q_{oi} - Q_i)/RT \tag{24}$$

From the different experimental figures, plotted in accordance with eq. (3) and eq. (4), and by means of eq. (16), (18), (19) and (20) the functions

$$-\ln X_{H_3O+} = f\left(\frac{Q_{H_3O+} - Q_{oH_3O+}}{RT}\right)$$

$$-\ln X_{Na+} = f\left(\frac{Q_{oH_3O+} - Q_{Na+}}{RT}\right)$$

have been calculated for the different electrodes at different conditions. As can be seen from the diagrams, Figs. 14—17, the requirements of eq. (24) are fullfilled, *i.e.* the experimental results are in agreement with the theoretical equation:

From the distribution law we have:

$$X_i = \frac{a_i}{\Sigma_i C_{og}} \exp\ [(Q_i - EF)/RT]$$

and from this and eq. (24):

$$X_i^{(1 + k_4)/k_4} = \frac{a_i}{\Sigma_i C_{og}} \cdot \frac{\exp\ (Q_{oi}/RT)}{\exp\ (FE/RT)} \tag{25}$$

and $$X_{H_3O+} = \left(\frac{a_{H_3O+}}{\Sigma_i C_{og}} \cdot \frac{\exp\ (Q_{oH_3O+}/RT)}{\exp\ (FE/RT)}\right)^{k_{4H_3O+}/(1 + k_{4H_3O+})} \tag{25a}$$

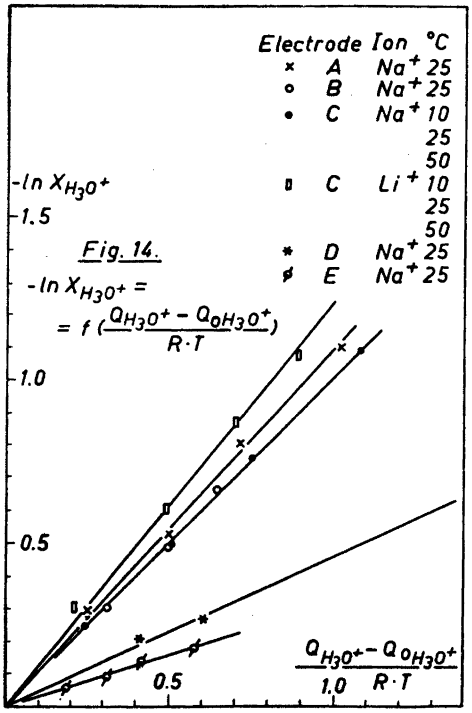
$$X_{Na+} = \left(\frac{a_{Na+}}{\Sigma_i C_{og}} \cdot \frac{\exp\ (Q_{oNa+}/RT)}{\exp\ (FE/RT)}\right)^{k_{4Na+}/(1 + k_{4Na+})} \tag{25b}$$

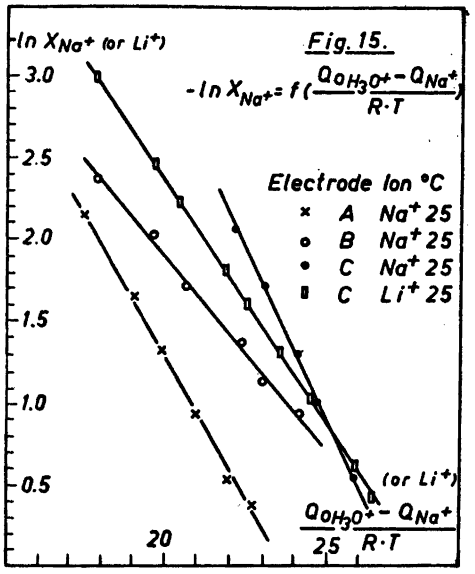
Let $(1 + k_{4H_3O+})/k_{4H_3O+} = \vartheta$ and $(1 + k_{4Na+})/k_{4Na+} = \tau$; and we get from eq. (25a) and eq. (25b):

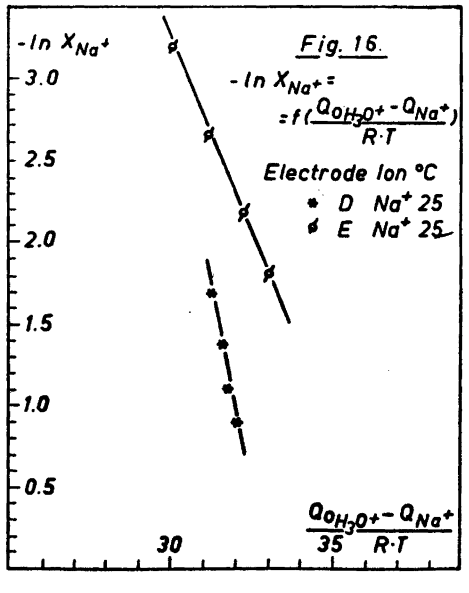
$$\frac{X_{H_3O+}^{\vartheta}}{X_{Na+}^{\tau}} = \varkappa \cdot \frac{a_{H_3O+}}{a_{Na+}} \tag{26}$$

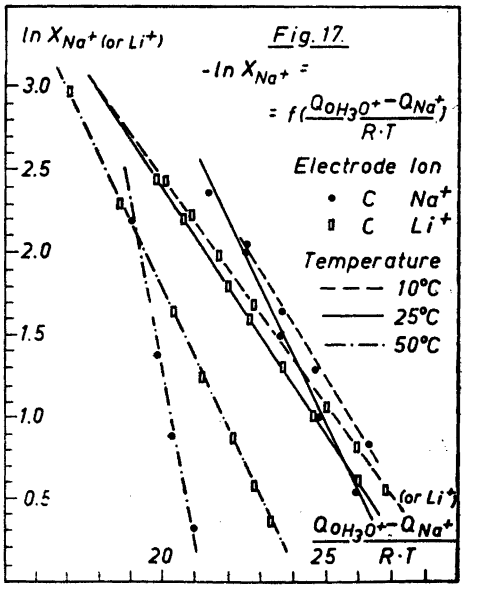
In this treatment eq. [26) is therefore the adsorption isotherm of the glass electrode. As $\Sigma\ X_i = 1$, the general equation for a system containing two different univalent ions can be written:

$$\Sigma \left(\frac{\exp\ (Q_{oi}/RT)}{\Sigma_i C_{og}} \cdot \frac{a_i}{\exp\ (FE/RT)}\right)^{k_{4i}/(1 + k_{4i})} = 1 \tag{27}$$


Electrode Ion °C
× A Na+ 25
o B Na+ 25
• C Na+ 10
25
50
▯ C Li+ 10
25
50
* D Na+ 25
ø E Na+ 25
-ln X_H3O+
1.5
1.0
0.5
Fig. 14.
-ln X_H3O+ =
= f ((Q_H3O+ - Q_oH3O+)/R·T)
0.5
(Q_H3O+ - Q_oH3O+)/R·T
1.0


ln X_Na+ (or Li+)
Fig. 15.
-ln X_Na+ = f ((Q_oH3O+ - Q_Na+)/R·T)
3.0
2.5
2.0
1.5
1.0
0.5
Electrode Ion °C
× A Na+ 25
o B Na+ 25
• C Na+ 25
▯ C Li+ 25
(or Li+)
20
(Q_oH3O+ - Q_Na+)/R·T
25


-ln X_Na+
Fig. 16.
-ln X_Na+ =
= f ((Q_oH3O+ - Q_Na+)/R·T)
Electrode Ion °C
* D Na+ 25
ø E Na+ 25
3.0
2.5
2.0
1.5
1.0
0.5
30
(Q_oH3O+ - Q_Na+)/R·T
35


ln X_Na+ (or Li+)
Fig. 17
-ln X_Na+ =
= f ((Q_oH3O+ - Q_Na+)/R·T)
Electrode Ion
• C Na+
▯ C Li+
Temperature
10°C
25°C
50°C
3.0
2.5
2.0
1.5
1.0
0.5
(or Li+)
20
(Q_oH3O+ - Q_Na+)/R·T
25

(This treatment probably cannot be applied in its simple form to multi-ion systems, as the adsorption sites not occupied by the ion i can be occupied by more than one kind of ion. This makes the treatment of the interaction energies more complicated. However, in practice the case of two univalent ions is of most interest.)

Applied to H_3O^+ and Na^+ eq. (27) will be, if $p = 1/\vartheta$ and $q = 1/\tau$:

$$\left(\frac{\exp(Q_{oH_3O+}/RT)}{\Sigma_i C_{og}} \cdot \frac{a_{H_3O+}}{\exp(FE/RT)}\right)^p + \left(\frac{\exp(Q_{oNa+}/RT)}{\Sigma_i C_{og}} \cdot \frac{a_{Na+}}{\exp(FE/RT)}\right)^q = 1 \quad (28)$$

This equation can be transformed:

$$-p \cdot pH + p \cdot Q_{oH_3O+}/M \cdot RT - p \cdot F \cdot E/M \cdot RT - p \cdot \log \Sigma_i C_{og} =$$
$$\log\left[1 - K\left(\frac{a_{Na+}}{\exp(FE/RT)}\right)^q\right] \text{ where } K = \left(\frac{\exp(Q_{oNa+}/RT)}{\Sigma_i C_{og}}\right)^q.$$

Since $-pH = FE'/MRT - Q_{oH_3O+}/MRT + \log \Sigma_i C_{og}$; and as $\Delta E = E - E'$ therefore

$$\Delta E = -\frac{MRT}{pF} \cdot \log\left[1 - K\left(\frac{a_{Na+}}{\exp(FE/RT)}\right)^q\right] \quad (29)$$

or

$$\Delta pH = -\frac{1}{p} \cdot \log\left[1 - K\left(\frac{a_{Na+}}{\exp(FE/RT)}\right)^q\right] \quad (30)$$

It is interesting to see that by expanding eq. (30) in

$$\log(1-x) \sim -x \ldots\ldots\ldots$$

we obtain

$$\Delta pH \sim \frac{K}{p}\left(\frac{a_{Na+}}{\exp(FE/RT)}\right)^q$$

and from this:

$$\log \Delta pH = q \cdot (pH_{measured} + \log a_{Na+}) + \text{constant} \quad (31)$$

and, with the approximation $a_{Na+} = \text{constant} \cdot C^a_{Na+}$:

$$\log \Delta pH = a \cdot pH_{measured} + b \cdot \log C_{Na+} + \text{constant} \quad (32)$$

This is the same as Jordan's empirical equation [21, 22].

Eq. (29) can also be written:

$$\frac{1}{\exp(pF\Delta E/RT)} = 1 - \left[\frac{\exp[(Q_{oNa+} - Q_{oH_3O+})/RT]}{\exp(F\Delta E/RT)} \cdot \frac{a_{Na+}}{a_{H_3O+}}\right]^q \quad (33)$$

As, in accordance with eq. (24)

$$k_4 = \frac{\ln X_i}{(Q_{oi} - Q_i)/RT}$$

is $k_4 = \infty$ if not the interaction is considered. In such a case $p = q = 1$, and from eq. (33):

$$\log\,[\exp(F\triangle E/RT) - 1] = \mathrm{pH} + \log\, a_{Na+} \cdot \exp[(Q_{oNa+} - Q_{oH_3O+})/RT] \quad (34)$$

This is the same as eq. (1) derived by Dole, neglecting interaction. (Eq. (1) contains C_{Na+} as Dole starts from what has been defined as ${}_iU_s$ in this paper, instead of the constant value ${}_iU_{os}$.) As mentioned earlier, the lack of agreement is that the experimental alkaline error figures do not give the slope of unity, as the equation requires.

As, in accordance with the following

$$\exp\,[(Q_{oNa+} - Q_{oH_3O+})/RT] \cdot \frac{a_{Na+}}{a_{H_3O+}} = \beta$$

$$F \cdot \triangle E/RT = x$$

eq. (33) can be written:

$$\frac{1}{e^{px}} = 1 - \frac{\beta^q}{e^{qx}} \text{ or } e^{qx} - e^{(q-p)x} = \beta^q$$

and after expanding in series and approximating, we get:

$$e^{qx} - e^{(q-p)x} \sim p \cdot x$$

However, this is also the first member of the expression for $p(e^x - 1)$, and we get:

$$p(e^x - 1) \sim \beta^q$$

And from this

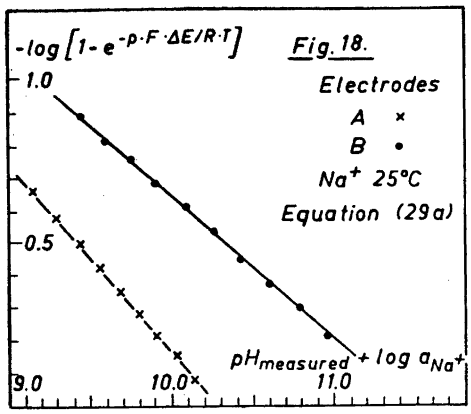
$$\frac{1}{q} \cdot \log[\exp(F\triangle E/RT) - 1] = \mathrm{pH} + \log\, a_{Na+} \cdot \exp[(Q_{oNa+} - Q_{oH_3O+})/RT] + \frac{1}{q} \cdot \log\, p \quad (35)$$

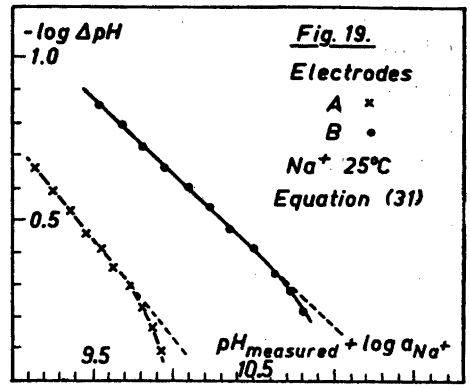
This equation is the same as eq. (1), except that it does not demand a slope of unity, and so can be brought into agreement with the experimental results.

Eq. (29) can also be written:

$$\frac{1}{q} \cdot \log[1 - \exp(-pF\triangle E/RT)] = \mathrm{pH} + \log\, a_{Na+} + \log\, K \quad (29a)$$

From the results obtained for the electrodes A and B, a check on the validity of eq. (29a), (31) and (35) can be obtained from Figs. 18—20. (The values of p are calculated from Fig. 14.) As can be expected from the approximations introduced, the agreement is good for low and moderate alkaline errors, but deviations appear at higher values. Deviations of the same kind can also be found in Dole's investigations, *e.g.* Dole [12], p. 271, Fig. 16.8. In the case of eq. (29a), where no mathematical approximations are introduced, it can be found that the equation is valid also at high alkaline errors, so that it describes the experimental behaviour of the glass electrodes in alkaline solutions.

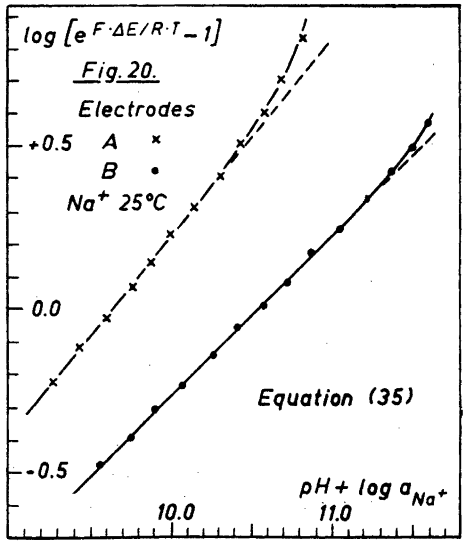


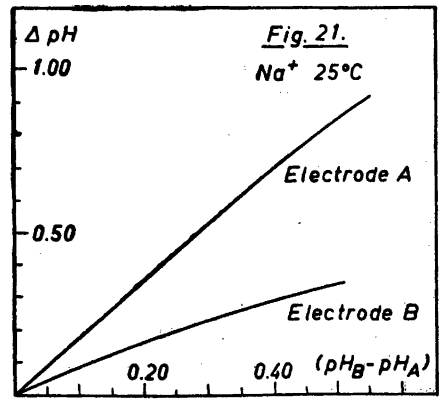


DISCUSSION

In the treatment given above, it has been shown that when the interaction in the surface layer is taken into account, an alkaline error equation can be derived, which is in good agreement with experimental results. The equation can also be approximated to the empirical equation suggested by Jordan, and to an equation of the Dole type, but with a slope in accordance with experimental data. It has also been shown that the interaction can be expressed as the sum of contributions of nearest neighbours. An adsorption isotherm for the glass electrode has been deduced, and the asymmetry potential given thermodynamic significance.

A more exact treatment of the significance of "contributions of nearest neighbours" would of cause be of great interest, but at present there is in-





sufficient theoretical and experimental background. The adsorption isotherm derived is probably of interest for ion exchange resins, where studies on different resins could give an indication of the interaction on a more general basis.

In the case of the glass electrode it would be of interest to determine the absolute amount of adsorbed ions, for example by means of radioactive isotopes. However, this approch is also difficult since it cannot be assumed that *e.g.* tritium is adsorbed to the same extent as protium, and furthermore the determination of tritium is very complicated. Other difficulties arise from the probable isotope equilibrium with water and the fact that the removal of excess solution from the glass will probably disturb the adsorption equilibrium.

However, some interesting figures can be obtained from the potential measurements. If in Figs. 15—17 an extrapolation is made to $-\ln X_{Na+} = 0$, *i.e.* $X_{Na+} = 1$, it is possible to determine:

$$(Q_{oH_3O+} - Q_{oNa+})/RT$$

This quantity is essentially the energy involved in the dissociation, so that a kind of dissociation constant, K_o, can be found from the formula:

$$(Q_{oH_3O+} - Q_{oNa+})/RT = -\ln K_o$$

or

$$pK_o = (Q_{oH_3O+} - Q_{oNa+})/M \cdot RT \qquad (36)$$

It may be expected that pK_o will be of the same order as the pH at which the alkaline error appears. pK_o can be considered as a characteristic of the glass electrode. Other characteristics are the interaction constants k_{4H_3O+} and k_{4Na+} in eq. (24) and these and pK_o will completely define the alkaline behaviour of the electrode. In Table 1 the values in question can be found for the electrodes studied. It would be of a great interest to determine these characteristics as functions of the electrode glass composition, and so facilitate the search for suitable glass electrode glasses.

A DIFFERENTIAL MEASURING METHOD

In the Swedish Patent 129833 a method is described for correcting the alkaline error in pH measurements when the activity of the sodium ion (or other) is unknown. The principle is that the measurements are made with two glass electrodes of different, but known, alkaline error functions. From the

Table 1. Characteristic constants of some glass electrodes.

Electrode and ion.	Temperature C°	pK_o	k_{4H_3O+}	k_{4Na+}
A Na+	25	10.4	1.08	0.36
B Na+	25	11.3	1.00	0.24
C Na+	10	11.6	1.00	0.32
C Na+	25	11.3	1.00	0.44
C Na+	50	10.1	1.00	2.00
C Li+	10	12.6	1.25	0.27
C Li+	25	12.2	1.25	0.32
C Li+	50	10.5	1.25	0.42
D Na+	25	14.3	0.41	1.00
E Na+	25	15.5	0.32	0.48

difference in measured pH for the two electrodes, pH_1—pH_2, the alkaline error for each can be found from a predetermined diagram. If curves similar to Fig. 2 are plotted from experimental data for the electrodes, each value of $M \cdot pH + \ln a_{Na+}$ corresponds to the alkaline errors ΔpH_1 and ΔpH_2, and as pH_1—$pH_2 = \Delta pH_2 - \Delta pH_1$ the required functions

$$pH_1 - pH_2 = f(\Delta pH_1) = g(\Delta pH_2)$$

can be determined. Such functions for the electrodes A and B are shown in Fig. 21.

The method seems to be valuable as highly stable low alkaline error electrodes seems to be rare, and for high temperature measurements, where even low alkaline error electrodes show a considerable deviation from ideal behaviour, and also when such electrodes are used for pH determinations in a range of high alkalinity. The method is also of interest when measuring at low temperatures, where the electrical resistance of some of said electrodes is rather high.

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Equilibria in Systems with Polynuclear Complex Formation

III. Derivation of a Generalized Bodländer Formula and a General Method of Calculating Equilibrium Constants

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A number of relationships have been derived which apply to equilibrium mixtures of two reactants, A and B, and their condensation products, of the general type A_pB_q. Denoting total, stoichiometric concentrations with capital letters, and equilibrium concentrations with small letters, the following relationship, concisely stated in Jacobian notation, is found to be universally valid:

$$J(B,\ \ln\ b/\ln\ a,\ A) \equiv \frac{\partial(B,\ \ln\ b)}{\partial(\ln\ a,\ A)} = 1$$

Solving for $\alpha = a/A$, the following equation results

$$\log\ \alpha = \left[\int_0^B (\partial\ \log\ \beta/\partial A)_B dB\right]_A$$

From this equation, α and hence a, can be calculated graphically from a set of known values of A, B and $\beta = b/B$. The equations presuppose constant activity factors, an assumption which is approximately correct for solutions of high, and constant ionic strength.

In the latter part of the paper a straightforward method is presented which can be used to find the composition of poly- and mononuclear complexes and the corresponding equilibrium constants from experimental data on any set of equilibria between two reactants.

The methods presented in this paper have been successfully applied by the author in a study on the hydrolysis of the iron(III) ion, Fe^{3+}.

This paper is number three in a series dealing with the problem of interpreting experimental data on polynuclear complex formation. In the two previous papers (AB I[1], AB II[2]) Sillén has worked out in detail the mathematics of so-called "core and links" mechanisms. He derived very useful and simple criteria by means of which data on polynuclear complex formation can be tested. Although the methods developed in AB I and AB II seem to be of far-

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reaching applicability, they do not cover all possible types of complex formation. Sillén purposely restricted his investigation to the "core and links" type of complex, which he has found to be rather common.

The field of polynuclear complex chemistry is in its infancy and it is, therefore, difficult to know which of several possible methods of interpreting experimental data will be the most useful in the majority of cases met with in practice. The accuracy of experimental data is, indeed, limited. One may therefore question if it is at all possible, at the present time, to prove the existence of more than a very limited number of species in a complex forming system, or, to put it another way, one may ask if experimental data, even in a favorable case, can supply more information than that represented by numerical values of, say three or four equilibrium constants. If this is so, it might, in an actual case, by very difficult to know exactly what is proof of the existence of the different possible complexes and what is merely a convenient method of correlating the experimental data in terms of the law of mass action. The methods in AB I and AB II should in many cases be useful for rejecting faulty hypotheses and also in choosing the one which is likely to be most consistent with the measurements.

In this paper a somewhat different method of attack is used in an effort to develop, in principle, a generally valid method of interpreting data on the formation of polynuclear complexes of the general type A_pB_q. The leading principle in the present work can be stated in the following way: No *a priori* assumption, whatsoever, is made as to the compositions or abundance of the complexes formed. This implies that the compositions of the complexes and the corresponding equilibrium constants will be obtained directly from a straight-forward method of calculation. In the author's opinion, the methods here presented should be of value as a supplement or an alternative to the methods in AB I and AB II.

The first part of the paper is devoted to the problem of calculating the equilibrium concentration of one of the reactants from a series of known values of the equilibrium concentration of the other and the total concentrations of the two reactants. Very often it is not possible to measure the equilibrium concentrations of both reactants directly. It is obvious that in such a case the interpretation of the data is made simpler if the unknown concentration can first be calculated, without presupposing any particular reaction mechanism.

In the second part of the paper, a method of interpretation of equilibrium data is presented which should be applicable to any type of mono- or polynuclear complex system, *provided data of sufficient accuracy are available.*

This paper is mathematical in character. The author has purposely omitted detailed discussions or examples of applications in order that the style should be as concise and as simple as possible. The methods presented here have been successfully applied in the author's investigation on the hydrolysis of the iron(III) ion, Fe^{3+}. The reader is referred to this work [3] for a detailed example of the application of the methods given in this paper.

List of symbols

A = total, stoichiometric concentration of the reactant A
a = equilibrium concentration of A
B = total, stoichiometric concentration of the reactant B
b = equilibrium concentration of B
$F_{q+1} = (F_q - I_q)/ab$
$I_q = q \sum_p \varkappa_{pq} a^{p-1}$
p = number of A atoms in the complex A_pB_q
q = number of B atoms in the complex A_pB_q
S = complexity sum (4)
x, y = arbitrary, independent variables, used in equations 10 and 10 a (see text)
$\alpha = a/A$
$\beta = b/B$
$\varkappa_{pq}$ = equilibrium constant for the reaction $pA + qB = A_pB_q$

DERIVATION OF A GENERALIZED BODLÄNDER'S FORMULA

If possible, emf-methods are generally preferred in the study of complex formation. The reasons are that they are accurate and relatively simple from the experimental point of view. A few recent modifications of the emf-method for studying complex equilibria have been of great importance in simplifying both the experimental technique and the interpretation of the data obtained.

These modifications are: Activity factors are kept constant by working with solutions of high, constant ionic strength. The equilibrium solutions under study are prepared by a titration technique [4]. In this way a large amount of experimental data can be obtained in a relatively short time and with a minimum of manipulation. By using two (in some cases three) burets during an emf-titration, the total, stoichiometric concentration of one of the reactants can be kept constant, while the other is varied. A series of such titrations at different total, stoichiometric concentrations of one of the reactants often covers the whole range of complexes of the two reactants, and is very convenient for the mathematical interpretation of the data.

The aim of the following reasoning and deductions is to show how the equilibrium concentration of one of the reactants is fixed by the other in all circumstances, and how it can be calculated from data of the kind mentioned above, without presupposing any particular reaction mechanism.

In the case of a *mono*nuclear process the solution of this problem is the well-known Bodländer formula [4] (see eq. 14 below).

The formulas derived below may be considered as extensions of this formula. In contrast to Bodländer's formula they are, however, applicable to any set of mono- and *poly*nuclear complexes.

Consider an equilibrium involving two reactants A and B, forming a condensation product A_pB_q:

$$pA + qB \rightleftharpoons A_pB_q$$

In the special case of an equilibrium between a metal ion and a complex forming substance, B may symbolize the metal ion and A the ligand. A set

of equilibria of this kind may exist simultaneously, since p and q may, in general, have any positive, integral values.

On applying the law of mass action to these equilibria, equations of the following general type are obtained:

$$\frac{[A_pB_q]}{a^p b^q} = \varkappa_{pq} \tag{1}$$

where $\varkappa_{pq}$ denotes the equilibrium constant, and a and b the equilibrium concentrations of A and B, respectively. Activity coefficients are assumed to be constant, as a consequence of working in a medium of high, and constant ionic strength; the law of mass action will therefore apply to concentrations as well as to activities. From stoichiometric considerations

$$A = a + \Sigma\, p\varkappa_{pq}\, a^p b^q \tag{2}$$

$$B = b + \Sigma\, q\varkappa_{pq}\, a^p b^q \tag{3}$$

where A and B denote total, stoichiometric concentrations. The summations have been carried out over all pairs of values of p and q except (1;0) and (0;1). The absence of competitive complex-formation with any third reagent C is assumed.

From now on the problem is a purely mathematical one. The method adopted by the author in order to find the universal relationship between a and b, consists of an elimination of the unknowns p, q, and $\varkappa_{pq}$ by successive, partial differentiation.

Let us first define the function S

$$S = \Sigma\, \varkappa_{pq}\, a^p b^q \tag{4}$$

Sillén [1] introduced the term "complexity sum" for this expression. Partial differentiation of (4) with respect to ln a and ln b gives

$$\left(\frac{\partial S}{\partial \ln a}\right)_b = a\left(\frac{\partial S}{\partial a}\right)_b = \Sigma\, p\varkappa_{pq}\, a^p b^q \tag{5}$$

$$\left(\frac{\partial S}{\partial \ln b}\right)_a = b\left(\frac{\partial S}{\partial b}\right)_a = \Sigma\, q\varkappa_{pq}\, a^p b^q \tag{6}$$

Substituting into equations (2) and (3)

$$\left(\frac{\partial S}{\partial \ln a}\right)_b = A - a \tag{7}$$

$$\left(\frac{\partial S}{\partial \ln b}\right)_a = B - b \tag{8}$$

A second differentiation of (7) with respect to ln b and of (8) with respect to ln a, and application of the identity

$$\left(\frac{\partial^2 S}{\partial \ln a\ \partial \ln b}\right) = \left(\frac{\partial^2 S}{\partial \ln b\ \partial \ln a}\right)$$

finally gives

$$\left(\frac{\partial B}{\partial \ln a}\right)_b = \left(\frac{\partial A}{\partial \ln b}\right)_a \tag{9}$$

At this point the unknowns p, q, and $\varkappa_{pq}$ have been eliminated.

Equation (9) is not yet very useful in the calculation of a from b, or *vice versa*, since a and b cannot be expressed explicitly. It may, however, be transformed to useful relationships by a transformation of variables. As in the case of the usual thermodynamic functions, the most convenient way of performing this transformation is by means of the Jacobian (functional) determinant [5,6]. In terms of Jacobians, equation (9) can be written

$$\frac{\partial(\mathrm{B}, \ln b)}{\partial(\ln a,\ \ln b)} = \frac{\partial(A, \ln a)}{\partial(\ln b,\ \ln a)} \tag{9a}$$

from which we get the most concise, basic form of the universal relationship between the variables involved

$$J(B,\ \ln\ b/\ln\ a,\ A) \equiv \frac{\partial(B,\ \ln\ b)}{\partial(\ln\ a,\ A)} = 1 \tag{9b}$$

If two arbitrary quantities x and y are chosen as independent variables, it follows from (9b)

$$\frac{\partial(B,\ \ln\ b)}{\partial(x,\ y)} = \frac{\partial(\ln\ a,\ A)}{\partial(x,\ y)} \tag{10}$$

or in determinant notation

$$\begin{vmatrix} \left(\frac{\partial B}{\partial x}\right)_y & \left(\frac{\partial B}{\partial y}\right)_x \\ \left(\frac{\partial \ln b}{\partial x}\right)_y & \left(\frac{\partial \ln b}{\partial y}\right)_x \end{vmatrix} = \begin{vmatrix} \left(\frac{\partial \ln a}{\partial x}\right)_y & \left(\frac{\partial \ln a}{\partial y}\right)_x \\ \left(\frac{\partial A}{\partial x}\right)_y & \left(\frac{\partial A}{\partial y}\right)_x \end{vmatrix} \tag{10a}$$

As an illustration, let $x = \ln a$ and $y = \ln b$. We then get from equation (10)

$$\left(\frac{\partial B}{\partial \ln a}\right)_b = \left(\frac{\partial A}{\partial \ln b}\right)_a$$

which is identical with equation (9). If we focus attention on the five quantities A, B, $\ln a$, $\ln b$, and $\beta = b/B$, ten pairs of independent variables are possible. For illustrative purposes a few of these cases have been worked out below. It is a simple matter to derive formulas based upon other sets of independent variables.

Special choices of independent variables

With A *and* B as independent variables, (10) takes the form

$$\left(\frac{\partial \ln b}{\partial A}\right)_B = \left(\frac{\partial \ln a}{\partial B}\right)_A \tag{11}$$

On introducing $\alpha = a/A$, and $\beta = b/B$, instead of a and b, we get from (11)

$$\left(\frac{\partial \ln \beta}{\partial A}\right)_B = \left(\frac{\partial \ln \alpha}{\partial B}\right)_A \tag{11a}$$

With *B and ln a* as independent variables (10) gives

$$\left(\frac{\partial \ln b}{\partial \ln a}\right)_B = -\left(\frac{\partial A}{\partial B}\right)_a \tag{12}$$

If we assume that all complexes are *mononuclear in* B, thus can be written as BA_n, then

$$S = b\ \Sigma \varkappa_n a^n = b \cdot f(a) \tag{13}$$

and from (7) and (8)

$$A = a + baf'(a); \qquad B = b + bf(a)$$

$$\left(\frac{\partial A}{\partial B}\right)_a = \frac{af'(a)}{1 + f(a)} = \frac{A - a}{B} \tag{13a}$$

For complexes mononuclear in B, (12) thus takes the form

$$\left(\frac{\partial \ln b}{\partial \ln a}\right)_B = -\frac{A - a}{B} \tag{14}$$

which is identical with Bodländer's well-known formula; $\frac{A - a}{B}$ is the average ligand number, which is often denoted by $\bar{n}$; in AB I and AB II it was denoted Z.

Choosing *A and ln b* as independent variables (10) gives an equation of the same form as (12)

$$\left(\frac{\partial \ln a}{\partial \ln b}\right)_A = -\left(\frac{\partial B}{\partial A}\right)_b \tag{15}$$

With *B and ln b*, we find

$$\left(\frac{\partial \ln a}{\partial B}\right)_b \left(\frac{\partial A}{\partial \ln b}\right)_B - \left(\frac{\partial A}{\partial B}\right)_b \left(\frac{\partial \ln a}{\partial \ln b}\right)_B = 1 \tag{16}$$

With *B and β* as independent variables, (10a) yields

$$\left(\frac{\partial A}{\partial \ln \beta}\right)_B \left(\frac{\partial \ln a}{\partial B}\right)_\beta - \left(\frac{\partial \ln a}{\partial \ln \beta}\right)_B \left(\frac{\partial A}{\partial B}\right)_\beta = 1 \tag{17}$$

Choosing *ln a and β* as independent variables, one gets

$$\left(\frac{\partial A}{\partial \beta}\right)_a + \left(\frac{\partial \ln b}{\partial \ln a}\right)_\beta \left(\frac{\partial B}{\partial \beta}\right)_a - \left(\frac{\partial B}{\partial \ln a}\right)_\beta \left(\frac{\partial \ln b}{\partial \beta}\right)_a = 0 \tag{18}$$

Finally with *ln b and β* as independent variables, one gets from (10a)

$$\left(\frac{\partial B}{\partial \beta}\right)_b - \left(\frac{\partial \ln a}{\partial \beta}\right)_b \left(\frac{\partial A}{\partial \ln b}\right)_\beta + \left(\frac{\partial A}{\partial B}\right)_b \left(\frac{\partial \ln a}{\partial \ln b}\right)_\beta = 0 \tag{19}$$

DISCUSSION

It should be pointed out that the equations derived in the preceeding paragraph apply to any equilibrium mixture of the two reactants A and B, forming one or more condensation products of the general type A_pB_q. A and B may, for instance, be two organic compounds, or hydroxyl ion and a metal ion, forming a series of mono- and polynuclear complexes.

Usually one knows from experiments at least three of the four variables involved, for instance A, B, and b. One may then wish to find a also, before proceeding to interpret the data in terms of equilibrium constants for the a priori unknown equilibria.

Equation (11), or its equivalent (11a), seems to be most suitable to the calculation of a, since it can be expressed explicitly in terms of the other variables by this equation.

As equation (11) is a partial differential equation, it is satisfied by an infinite number of functions b (A, B, a) or a (A, B, b). The function a sought is the one which satisfies both (11) and the boundary conditions, as given by the experimental data. There are at least two generally valid boundary conditions

$$\ln \alpha = 0, \text{ for } B = 0 \tag{20a}$$

and

$$\ln \beta = 0, \text{ for } A = 0 \tag{20b}$$

which are independent of the actual reaction mechanism. On account of the symmetrical nature of (11), we shall consider only the first one. Solving equation (11a) for $\log \alpha$ gives

$$\log \alpha = \left[\int_0^B \left(\frac{\partial \log \beta}{\partial A} \right)_B \mathrm{d}B \right]_A \quad \text{or} \quad \log \frac{a}{A} = \left[\int_0^B \left(\frac{\partial \log \frac{b}{B}}{\partial A} \right)_B \mathrm{d}B \right]_A \tag{21a}$$

This relation suggests a relatively simple and straightforward method of calculating $\log \alpha$. For each series of experiments, at constant B, one plots $\log \beta$ against A, determines the derivatives of these curves at a certain A value, and finally the corresponding value of $\log \alpha$ from the area under $(\partial \log \beta / \partial A)_B$, when plotted against B.

Conversely, one may also calculate b from data of the form (A, B, a), applying the equation

$$\log \beta = \left[\int_0^A \left(\frac{\partial \log \alpha}{\partial B} \right)_A \mathrm{d}A \right]_B \tag{21b}$$

The graphical method discussed here suggests that measurements at a relatively large number of different values of B (or of A, respectively), including low values, are desirable in the calculation of a from b (or *vice versa*). This is one reason for trying to extend the range of concentrations as much as possible in investigating polynuclear equilibria.

For further details on the use of (21), the reader is referred to the author's paper on the hydrolysis of the iron(III) ion [3].

EVALUATION OF EQUILIBRIUM CONSTANTS OF A POLYNUCLEAR COMPLEX FORMATION PROCESS

In the evaluation of the equilibrium constants of a polynuclear process a knowledge of both a and b is highly desirable. If, therefore, only one of the two equilibrium concentrations can be measured directly, the other may be calculated by the methods developed in the preceeding section, which presupposes no particular reaction mechanism. When both a and b are known, the equilibrium constants may be calculated in a rather straightforward manner, as will now be shown.

Equation (3) may be written in the following way

$$F_1 = \frac{(B - b)}{ab} = \sum_{p,q} q\varkappa_{pq}\, a^{p-1}\, b^{q-1}$$

When $q = 1$, then F_1 will be a function of a only, independent of b. Bjerrum [7], Leden [4] and others have already given detailed accounts of the problem of dealing with mononuclear processes.

In order to evaluate the "degree" (q_{max}) of the polynuclear process, we may plot F_1 against b at a certain constant value of a. If a straight line with an intercept, I_1, and a finite slope is obtained, then only mono- and dinuclear complexes are present; if a parabola, trinuclear complexes are also present, and so on. It may be difficult to decide between curves of higher degrees than the first, but this problem can be solved in the following way.

From the plot of F_1 against b the intercept

$$I_1 = \sum_p \varkappa_{p\,1} a^{p-1}$$

is obtained. If then

$$F_2 = \frac{(F_1 - I_1)}{ab}$$

is plotted against b, the new intercept is given by

$$I_2 = 2 \sum_p \varkappa_{p\,2} a^{p-2}$$

This same procedure is repeated until a straight line is obtained. The "steps" ($q = 1,2,3 \ldots.$) of the process will be successively separated as intercepts:

$$I_q = q \sum_p \varkappa_{pq}\, a^{p-q}$$

$$F_{q+1} = \frac{F_q - I_q}{ab}$$

For each value of q, p can, in the general case, assume more than one value. In order to find all equilibrium constants, one must compare the F-curves obtained at different, constant values of a. From the resulting system of equations all $\varkappa_{pq}$'s may be calculated.

This method is laborious, but it appears to be the best method available, that does not involve any presuppositions as to the actual mechanism. In practice, the accuracy of the measurements is limited, and the data will probably be exhausted after the introduction of but a few constants. The calculations may therefore in reality become relatively simple. As an example, it may be mentioned that in the application of this method of calculation to the hydrolysis of Fe^{3+}, the data could be explained, within the limits of experimental error, by introducing only three constants, $\varkappa_{11}$, $\varkappa_{12}$ and $\varkappa_{22}$, corresponding to the formation of $FeOH^{2+}$, $Fe(OH)_2^{+}$ and $Fe_2(OH)_2^{4+}$, respectively.

On the other hand, it might happen that the actual reaction mechanism consists of a very large or even infinite number of steps ($q = 1 \ldots \infty$), as has been suggested [8] for the hydrolysis of Bi^{3+} and, more recently [9, 10], for Th^{4+} and UO_2^{2+}. This should be indicated by the fact that in such a case the data will not yield a straight line even for high orders (high q-values) of F_q, when plotted against a.

Acknowledgement. The author is much indebted to Professor L. G. Sillén for his advice and inspiring interest in the work.

Thanks are also due to Dr F. Rossotti, Oxford, for his valuable criticism of the paper.

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Note added in proof: Equation (21a), which was applied in 1952 to the iron(III) hydrolysis [3] is related to eq. (24) in a paper of McKay (*Trans. Faraday Soc.* **49** (1953) 237). McKay's equation may be obtained from (12) whereas (21a) is derived from (11).

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On Vitamins in Sewage Sludge

II *. Formation of Vitamin B_{12}-, Folic Acid-, and Folinic Acid Factors in Municipal Sludge

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The decomposition of municipal sewage sludge by means of anaerobic and aerobic fermentation was studied with respect to the content and distribution of vitamin B_{12}-, folic- and folinic acid factors.

Factor B was found to represent an intermediate step in the decomposition as well as in the synthesis of other vitamin B_{12} factors.

Factor C_2 seems to occur only during the early stages of the microbial decomposition of the sludge.

The quantitative estimation of vitamin B_{12} activity by different methods and test organisms is discussed in connection with the experiments performed.

A new factor active towards *Streptococcus faecalis* was shown to be present during early stages of the anaerobic fermentation of sludge and was found to be chromatographically identical with a synthetic factor.

The mechanism of the formation of the different vitamin B_{12}-factors during decomposition of sewage sludge is discussed.

It has been previously reported from this laboratory [1] and by other authors [2,2a] that digested sewage sludge contains considerable amounts of 4—6 different vitamin B_{12}-factors. Several folic acid and folinic acid factors have also been found in the sludge [1].

In order to ascertain the necessary statistical basis for the estimation of the quantitative distribution of vitamin B_{12}-activity amongst these factors [3], data have been collected from assays during the last three years. Though the values obtained in this way were fairly constant over long periods of time, considerable deviations could however be noted in a few cases. It happened for instance that in some sludge samples almost all of the vitamin B_{12}-activity was due to the factors B and C, or to the factors A and pseudovitamin B_{12}, and only to a small degree to cyanocobalamin. In order to find some explanation for these phenomenona an attempt was made to investigate in what way the

* The first paper in this series was published by Sjöström, A.G.M., Neujahr, Halina Y. and Lundin, H. *Acta Chem. Scand.* 7 (1953) 1036.

different vitamin B_{12}-factors are formed in the sewage sludge when it is fermented in different manners. For this purpose primary sludge from a settling tank was fermented both aerobically and anaerobically in the laboratory, samples being taken during the fermentation and analyzed for vitamin B_{12}-, folic acid-, and folinic acid-activities in both quantitative and qualitative respects.

Anaerobic digestion of sewage sludge is a very slow process when allowed to develop spontaneously without seeding with previously digested sludge. According to Imhoff and Fair [4] the digestion proceeds in three stages:

1) "Acid fermentation", characterized by intensive acid production (pH drops below 6.0) and putrefactive odours. This stage lasts about 2—3 weeks, large volumes of gas (chiefly CO_2 with some H_2S) being produced.

2) "Acid regression" — a lengthy period (about 3—4 months) with small volumes of gas produced (CO_2 + H_2) and slow rise of pH to about 6.8.

3) "Alkaline fermentation" — large volumes of gas (chiefly CH_4 + CO_2) are liberated. The pH-value may rise above 7.0 and the sludge becomes well buffered.

"Acid fermentation" and "acid regression" constitute a "breaking in" or "ripening" period through which all primary sludge which is not seeded must pass when fermented anaerobically. Once alkaline fermentation is established the well buffered sludge rich in enzymes and possesing proper bacterial flora exerts a controlling influence over the course of digestion of incoming fresh sludge.

In technical practice, therefore, digestion tanks already containing well-digested sludge are charged daily with fresh sludge in amounts that cannot upset the alkaline fermentation. At the sewage plants of Stockholm, for instance, only 1/30 of the digested sludge in the digestion tanks is daily replaced by fresh solids, the total time for sludge digestion being 60 days. Obviously, this method could not be used in our laboratory digestion experiments since the microbiological methods for the determination of vitamin B_{12} have limits of error of ± 20 %. These limits are probably still wider in the experiments where the vitamin B_{12}-activity is due to several different factors. Consequently we limited the amount of "seed sludge" to only 10 % in two experiments and in other experiments no seeding at all was used. The total time of fermentation was thus extremely prolonged.

EXPERIMENTAL

Fresh sludge from a settling tank of the municipal sewage plant of Stockholm was disintegrated in a "Turmix" blendor and placed in glass fermentors each of 10 liters capacity, provided with stirrers, heating coils, arrangements for inlet of gas and for removal of samples. The appropriate temperature (33° C or 55° C) was obtained by circulating warm water from constant temperature baths through the coils of the fermentors with simultaneous stirring. In anaerobic digestions, nitrogen gas was blown through the sludge until all air was removed, this procedure being used every time the sludge came in contact with air, *e. g.* after removing a sample. In the aerobic fermentation, air was blown through the sludge continously. The gas evolved in anaerobic digestions was collected and measured. Four parallel experiments were performed according to the scheme shown in Table 1.

Samples of about 100 ml were taken off at regular time intervals (3—4 days) and the pH and content of dry solids were determined. 30 ml of each sample was treated as follows:

100 μg NaCN for each ml sludge was added, pH adjusted with sulphuric acid to 5.5, the sample autoclaved at 120° C for 10 min. and centrifuged. The centrifugate was used for the following estimations:

1) vitamin B_{12}-activity with the cup plate, the tube, and the bioautographic methods using *Escherichia coli* 113-3 and *Lactobacillus leichmannii* 313 and 4797, respectively.

2) "folic acid" and "folinic acid" activities with the cup plate and bioautographic methods using *Streptococcus faecalis* and *Leuconostoc citrovorum*, respectively.

Table 1.

Experiment No.	Type of treatment	Temp.	Seeding	pH adjustment	Additions
1	anaerobic	33° C	with digested sludge, 10 % v/v ,at the start	with $Ca(OH)_2$ to 7.0, at the start	crystalline cyanocobalamin after 80 days in an amount corresponding to0.08 μg B_{12}/ml sludge *
2	anaerobic	33° C	none	with $(NH_4)_2CO_3$ to 7.0, after the period of acid fermentation,	no
3	anaerobic	55° C	with digested sludge, 10 % v/v, after 10 days	with $Ca(OH)_2$ to 7.0	no
4	aerobic	33° C	none	no	no

* The reason for this addition is explained on p. 626.

RESULTS AND DISCUSSION

Vitamin B_{12}-factors

In experiments 1, 2 and 4 considerable amounts of cyanocobalamin were formed during the later stages of fermentation. In experiment 3, on the contrary, a destruction of all vitamin B_{12}-factors after about 100 days could be noted. This was probably caused by the relatively high temperature in connection with the prolonged time of the experiment (*cf.* p. 626). In practice where seeding with a sufficient amount of well-digested sludge can be made, such a thermophilic digestion of sewage sludge is performed in only 10—15 days. The course of formation of vitamin B_{12}-factors in the four experiments is represented in Figs. 1 a, b, c, d, respectively, by means of bioautograms with *E. coli* 113-3.

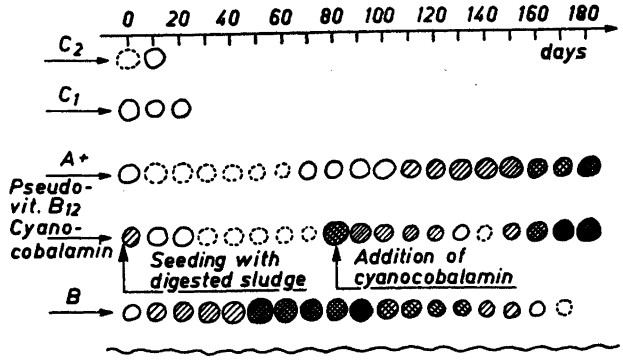

Fig. 1 a. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 33° C (with seeding).

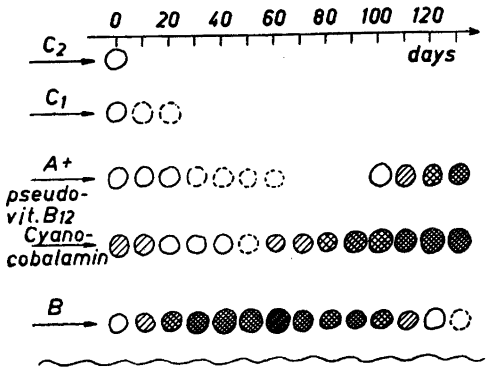

Fig. 1 b. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 33° C (without seeding).

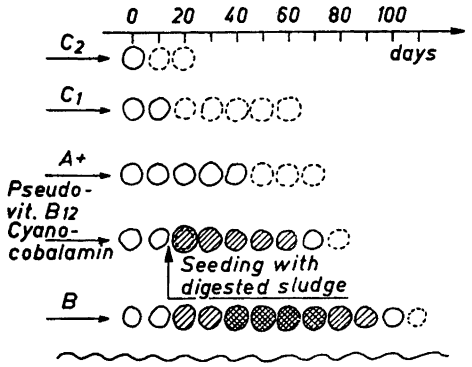

Fig. 1 c. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 55° C (with seeding).

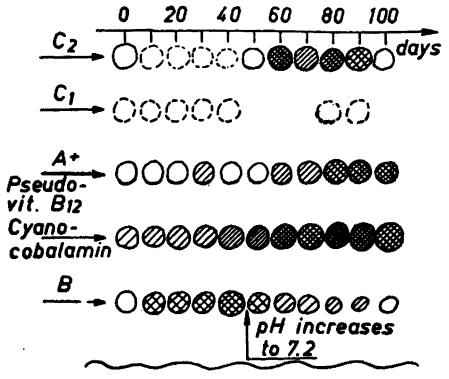

Fig. 1 d. Formation of vitamin B_{12}-factors during microbial decomposition of sewage sludge. Bioautogram. Aerobic fermentation at 33° C (without seeding).

It can be seen in Figs. 1 a and 1 b that in anaerobic fermentation the amounts of cyanocobalamin and of factors A + pseudovitamin B_{12} — present in the fresh sludge and the seeding — are usually destroyed during the first stage of digestion whilst factor B is simultaneously formed. To confirm this surprising observation, that cyanocobalamin is destroyed instead of being formed, crystalline cyanocobalamin was added after 80 days to the digestion tank of experiment 1 (Fig. 1 a). This amount of cyanocobalamin was also slowly destroyed. In the last stage of the digestion (after about 5—6 months) cyanocobalamin and factors A + pseudovitamin B_{12} were reformed while factor B disappeared. Factor C_1 and factor C_2 were present only at very early stages of the digestion.

Anaerobic digestion at 55° C (Fig. 1 c) had a similar course with initial destruction of cyanocobalamin and formation of factor B. At the later stage of this digestion, all vitamin B_{12}-factors were destroyed, probably due to the high temperature in connection with the prolonged time of the experiment as already mentioned. It can be seen in Fig. 1 c that factor B is comparatively more resistant to this destruction than the other factors which is in good agreement with the observation made above and by other authors[5,6] that this factor is a decomposition product of cyanocobalamin and factors A + pseudovitamin B_{12}.

In the aerobic fermentation of sludge (Fig. 1 d), no destruction of cyanocobalamin could be noted. This factor was instead formed continuously and, after 70 days, became the dominant form of vitamin B_{12}. Notable amounts of factor B were formed during the first 30 days, but after 40 days, when the pH-value increased to 7.0—7.5 (Figs. 1 d and 2 b), this factor slowly disappeared while considerable amounts of cyanocobalamin were formed, and also of factors A + pseudovitamin B_{12} and of factor C_2.

Quantitative estimation of vitamin B_{12}-activity. The quantitative estimation of vitamin B_{12} activity formed during the anaerobic and aerobic fermentation as performed by two different methods with *E. coli* 113-3, revealed a striking discrepancy between the values obtained (Figs. 2 a and 2 b).

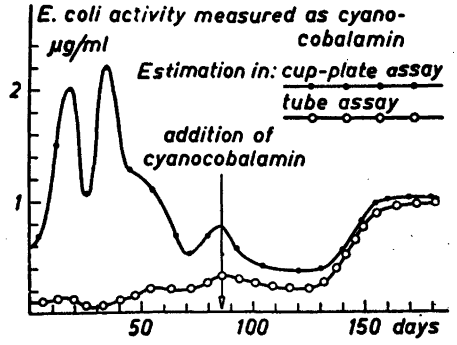

Fig. 2 a. Variations in vitamin B_{12}-activity during microbial decomposition of sewage sludge. Anaerobic fermentation at 33° C.

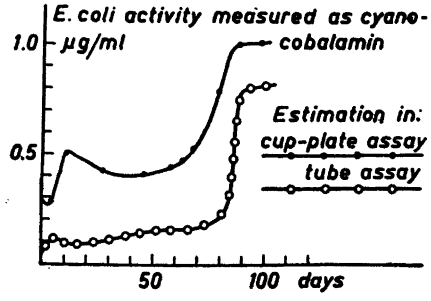

Fig. 2 b. Variations in vitamin B_{12}-activity during microbial decomposition of sewage sludge. Aerobic fermentation at 33° C.

Figs. 2 a and 2 b show that, in the determination of the vitamin B_{12} activity of sewage sludge, the cup-plate method gives much higher values than the turbidimetric method. This is due to the fact that the sludge contains not only cyanocobalamin but also other vitamin B_{12}-factors, many of which in the cup plate method exert a greater activity towards the test organism than in the tube method. The discrepancy in the values is especially pronounced when factors B and C are present in considerable amounts (*cf. e.g.* Figs 1a and 2a). Gregory and Holdsworth [7] have determined the activities of different vitamin B_{12}-factors towards *E. coli* in the tube assay and found them to be 34—92 % lower than that of cyanocobalamin. The corresponding activities in the cup-plate assay as found by Ford [8] are much higher than the activity of cyanocobalamin with differences varying between 274 and 1 000 %. This may account for the fact that the cup-plate method proved to be much less reliable than the turbidimetric method in the estimation of vitamin B_{12}-activity of sewage sludge, which often contains relatively large amounts of factors B, C and pseudovitamin B_{12}. However as soon as cyanocobalamin becomes the main vitamin B_{12}-factor in the sample analyzed, both methods may be of the same degree of reliability, as can be seen, for instance, in Fig. 2 a (activity values after 150 days of digestion) and in Fig. 1 a which shows that after 150 days cyanocobalamin and factors A + pseudovitamin B_{12} dominate.

A large discrepancy has also been found between the values for vitamin B_{12} activity obtained by assaying with *E. coli* 113-3 and *L. leichmannii*, the latter values being much lower. This is in agreement with the findings of Ford [9] that the "vitamin B_{12}-like" factors are much less active towards *L. leichmannii* than towards *E. coli* 113-3, factor B not being active at all towards the first mentioned organism.

Discussion. The formation of cyanocobalamin and other vitamin B_{12}-factors in sewage sludge is apparently a complicated process, proceeding in several stages and being caused by several different microorganisms, anaerobic as well as aerobic. Some of the anaerobic organisms probably consume cyanocobalamin and produce factor B which is then transformed by other organisms to cyanocobalamin and to factors A + pseudovitamin B_{12}. Factor C_2 seems to occur only during the growth period of the microorganisms. It was present during the early stages of both the anaerobic and the aerobic fermentations. In the aerobic fermentation it was also found at a later stage where the pH increases from about 5.0 to about 7.2 probably allowing a new microflora to grow.

However the results obtained until now do not exclude the possibility that during anaerobic fermentation the destruction of factors $C_1 + C_2$ at an early stage and the more slowly proceeding destruction of cyanocobalamin and factors A + pseudovitamin B_{12} during formation of factor B, and the disappearance of factor B under reformation of cyanocobalamin and factors A + pseudovitamin B_{12} during a later stage are independent processes, caused by different microorganisms. The formation of these factors during the different stages of fermentation could thus be caused by successive changes in the sludge, allowing different microorganisms to grow and to produce their characteristic growth factors.

In view of the findings of Armitage *et al.*[5] and of Gant *et al.*[6] that factor B is cyanocobalamin, factor A, or pseudovitamin B_{12} lacking their respective nucleotides, it seems possible that factor B formed during the digestion of sludge may derive from the decomposition of these three factors, or some of them. It is, however, probable that at least some of the organisms active in sludge digestion may be able to directly synthesize factor B. This seems to hold especially for organisms active in the aerobic decomposition of sludge since in this case no destruction of cyanocobalamin occurred during the formation of factor B. The mechanism of formation of factors A + pseudovitamin B_{12} could not be explained with any degree of probability. For the formation of these two factors * the presence of both factor B and cyanocobalamin may be necessary; cf. Fig. 1 a and 1 b.

Probably, the best solution of these problems could be obtained by isolation of the different organisms active in sludge decomposition and by investigating their ability to synthesise the different vitamin B_{12}-factors. This task, however, has proved so difficult and time consuming that we had to postpone it for the time being, especially as we have some reason to suspect that complicated symbiotic and synenergistic phenomenona are involved in the mechanism of vitamin B_{12} formation in the sludge.

Folic acid- and folinic acid-factors.

The course of formation of these factors in the anaerobic fermentation of sewage sludge carried out at 33° C is represented in Fig. 3 a and 3 b.

Fig. 3 a shows that during the digestion the "folinic acid activity" diminishes much quicker than the "folic acid activity" does. Fig. 3 b shows a chromatographic picture of different factors present and active towards *S. faecalis*. At early stages of the digestion the formation of a *new S. faecalis*-factor — PFH — with R_F-value = 0.75 could be noted (see Fig. 3 b). This factor has been chromatographically identified with a synthetic factor obtained by Ericson[9] in this laboratory by formylation and reduction of pteroic acid. This factor could not be found in a wort fermented by an anaerob, *Clostridium thermocellulaseum*[10].

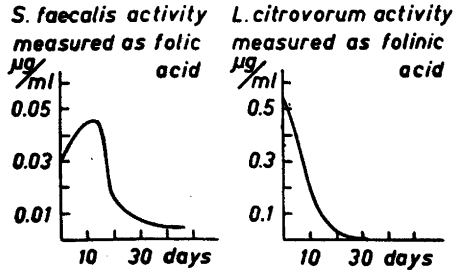

Fig. 3 a. Variations in S. faecalis- and L. citrovorum-activities during microbial decomposition of sewage sludge. Anaerobic fermentation at 33° C.

* After this paper had been prepared for publication, the latest papers of Ford and Holdsworth[9] came to our knowledge. In view of the synthesis of factor A, pseudovitamin B_{12} and cyanocobalamin, performed by these authors from factor B and precursors of different nucleotides, it seems likely that the formation of these three factors at a later stage of sludge decomposition depends on the appearance of the corresponding precursors in the medium.

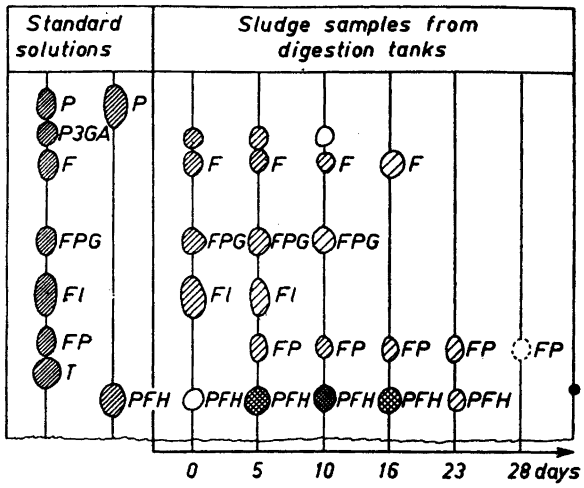

Fig. 3 b. Formation (destruction) of S. faecalis-factors during microbial decomposition of sewage sludge. Bioautogram. Anaerobic fermentation at 33° C.

P = *pteroic acid*
P3GA = *pteroyltriglutamic acid*
F = *folic acid*
FPG = *formylpteroylglutamic acid*
Fl = *folinic acid (Leucovorin)*
PF = *formylpteroic acid (Rhizopterin)*
PFH = *pteroic acid, formylated, hydrated.*

The appearance of this factor between the 5th and the 23rd day of digestion (Fig. 3 b) may explain the maximum of the *S. faecalis*-activity curve occurring on the 14—15th day of digestion (Fig. 3 a).

Bioautograms with *L. citrovorum* revealed activity mainly due to folinic acid (Leucovorin Lederle) and only to a less degree due to two unidentified factors with R_F-values 0.18 and 0.22. The presence of these factors in sludge has already been reported in a previous work [1]. Nothing of special interest concerning the course of formation or disappearance of these factors could be noted.

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The Kinetics of the Decarboxylation of *tert.*-Butylpropiolic Acid and its Sodium Salt in Water

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In order to determine to what extent the rates of decarboxylation of α,β-acetylenic acids are influenced by various structural effects, a kinetic study has been made of the decomposition of *tert.*-butylpropiolic acid and its sodium salt in water at six temperatures. The data obtained have been used to calculate values of the activation energy, frequency factor, free energy of activation, heat of activation and the entropy of activation. The rates of decomposition of *tert.*-butylpropiolic acid and its salt are approximately equal to those of tetrolic acid and its salt, but the activation energies for the former are of the same magnitude as those found for propiolic acid and its sodium salt. It has been concluded that the low rates of decomposition of tetrolic acid and its salt compared to those of propiolic acid and its salt are due to the occurrence of hyperconjugation in the former.

In previous work [1] on the decarboxylation of some α,β-acetylenic acids and their salts, it was established that the decompositions reactions of tetrolic acid ($CH_3—C\equiv C—COOH$) and its sodium salt are much slower than those of propiolic acid ($HC\equiv C—COOH$) and its salt. It seemed that this could be attributed to hyperconjugation in tetrolic acid. To determine the validity of this assumption, the decarboxylation of *tert.*-butylpropiolic acid and its sodium salt in aqueous solution has been studied. The reactions involved are the following:

$$(CH_3)_3C—C\equiv C—COOH \rightarrow (CH_3)_3C—C\equiv CH + CO_2$$

$$2\ (CH_3)_3C—C\equiv C—COONa + H_2O \rightarrow 2\ (CH_3)_3C—C\equiv CH + CO_2 + Na_2CO_3$$

EXPERIMENTAL

Pinacone hydrate and pinacolin. Pinacone hydrate was first synthesized according to the method given in the Organic Syntheses [2]. The product was then transformed into pinacolin by a method also given in Organic Syntheses [2].

Chlorination of pinacolin with phosphorus pentachloride [3]. 140 g of pinacolin was slowly added to 300 g of phosphorus pentachloride in a flask immersed in a mixture of

ice and water. The reaction mixture was then allowed to stand 4 hours at room temperature, after which it was gradually added to a mixture of water and ice. The precipitate formed was separated by filtration, washed with water and dried by suction. The liquid reaction product in the filtrate was separated from the water in a separating funnel and dried with calcium chloride. The yield of the solid product was 92 g, that of the liquid product 84 g.

Tert.-*butylacetylene* [4]. The chlorinated products of the preceding synthesis were heated six hours with 500 g of potassium hydroxide and 40 ml of ethanol in a graphite bath at 150—270° C. The liquid distilling from the mixture below 50° C was collected, washed with water, dried over potassium hydroxide and subjected to fractional distillation in a Widmer column. The yield of *tert.*-butylacetylene boiling at 37.5—38.5° was 55 g.

Sodium tert.-*butylacetylide* [5]. 23.8 g of the *tert.*-butylacetylene was dissolved in 150 ml of anhydrous ether in a flask fitted with an efficient reflux condenser to which a calcium chloride tube was attached. 6.5 g of thin sodium wire were added. The reaction was allowed to continue 24 hours at room temperature, the flask being shaken at intervals. The mixture was then boiled 18 hours until a sample no longer gave a precipitate on adding ammoniacal silver nitrate solution.

Tert.-*butylpropiolic acid* [5]. Carbon dioxide gas dried by passing it through concentrated sulphuric acid was led into the flask containing the ether solution of *tert.*-butylacetylide during 26 hours at room temperature with intermittent shaking, after which the mixture was boiled for six hours. During this time the grey precipitate of sodium *tert.*-butylacetylide turned white and a sample no longer gave off *tert.*-butylacetylene when water was added to it. The contents of the flask were carefully added to ice-water and the solution was extracted with ether after acidification with sulphuric acid. The combined ether solutions were dried with anhydrous sodium sulphate, and the ether removed by distillation. The rest of the *tert.*-butylacetylene was treated in a similar manner. The products were combined and distilled by fractionation. The yield of product boiling at 112.5—113.0° at 14 mm Hg was 42.7 g. Its melting point was 47.9—48.0°. Analysis by titration: 30.0 mg of the acid consumed 11.65 ml of 0.0202 *N* barium hydroxide, which corresponds to an equivalent weight of 127.48 (calc. 126.15). The odour of the acid resembled those of propiolic and tetrolic acids and the acid was very hygroscopic.

Tetrolic acid. The tetrolic acid was the same as that used in the previous work [1].

Methods. The apparatus and the methods used in the measurement of the rates of decomposition were the same as in the earlier investigation [1].

RESULTS

The rates of decomposition of *tert.*-butylpropiolic acid and its sodium salt were measured in aqueous solution at six temperatures. The rate of decomposition of sodium tetrolate was measured in aqueous solution at three temperatures to extend the temperature range of the earlier measurements [1]. Similarly as in the earlier work, the initial concentrations of the acids were 0.01 *M*, those of the salts 0.02 *M*.

The reactions studied were all of the first order. The specific rates of the salts showed a good constancy over the range 20—70 % reaction, as seen from the example in Table 1. The rate constants were computed from the equations employed earlier [1].

Table 1. The decarboxylation of sodium tert.-*butylpropiolate in aqueous solution at 130.19° C.*

Time in sec.	7 260	10 620	13 200	15 660	21 060	26 400	35 640
% reaction	22.8	31.4	38.0	42.8	52.8	61.0	72.0
$10^5 k$ sec.$^{-1}$	3.565	3.549	3.621	3.567	3.565	3.567	3.572

The plots of log k against $1/T$ gave excellent straight lines for both sodium *tert.*-butylpropiolate and sodium tetrolate, the relative maximum deviations of the experimental points from the line calculated by the method of least squares being approximately 1 %.

The rate constants for the decarboxylation of *tert.*-butylpropiolic acid were satisfactorily constant at the higher temperatures; at the lower temperatures the values tended to increase towards the end of the reaction. Also the plots of log k against $1/T$ deviated from the linear course at the lower temperatures, whereas the values for the four higher temperatures were situated close to the same straight line. For this reason, only the latter four values were employed in the calculation of the activation energy, and thus the value obtained is a maximum value. A similar deviation from the Arrhenius plot has not been observed for the decarboxylation reactions of other acetylenic acids that have been studied.

The values of the rate constants are collected in Table 2 and the values of derived kinetic quantities in Table 3. The values of E and A, the energy of activation and the frequency factor, have been computed by the method of least squares. The values of the free energy of activation ΔG^*, the heat of activation ΔH^* and the entropy of activation ΔS^* refer to 100° C.

Table 2. Decarboxylation rate constants.

Reactant	$10^7 k$ sec.$^{-1}$						
	106.78°	115.03°	122.44°	130.19°	137.33°	144.32°	152.65°
$(CH_3)_3C-C\equiv C-COOH$		34.6	69.6	142	270	501	1 010
$(CH_3)_3C-C\equiv C-COONa$	26.7	68.5	160	357	747	1 510	
$CH_3-C\equiv C-COONa$	28.8				903	1 840	

Table 3. Values of derived kinetic quantities.

Reactant	A sec.$^{-1}$	E cal.	ΔG^* cal.	ΔH^* cal.	ΔS^* E.U.
$(CH_3)_3C-C\equiv C-COOH$	$2.12 \cdot 10^{11}$	29 850	32 530	29 110	−9.1
$(CH_3)_3C-C\equiv C-COONa$	$8.03 \cdot 10^{13}$	33 870	32 140	33 120	+2.7
$CH_3-C\equiv C-COONa$	$3.51 \cdot 10^{14}$	34 920	32 090	34 180	+5.6

DISCUSSION

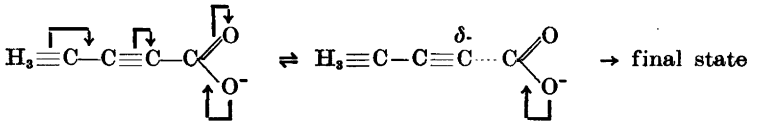
For comparison, the rate constants at 90° C, the activation energies, frequency factors and entropies of activation at 100° C for the decomposition of propiolic, tetrolic and *tert.*-butylpropiolic acids and their sodium salts are given in Table 4. It is seen that whereas sodium propiolate decomposes 25 times as rapidly as sodium tetrolate, sodium *tert.*-butylpropiolate decomposes at approximately the same rate as sodium tetrolate. The difference in the rates

Table 4. Kinetic data for the decarboxylation of propiolic, tetrolic and tert.*-butylpropiolic acids and their salts in aqueous solution.*

Reactant	$10^7 k_{90}{}^\circ$*	E cal.	A sec.$^{-1}$	ΔS* E.U.
$HC{\equiv}C-COONa$	82.8	33 610	$1.38.10^{15}$	+8.3
$CH_3-C{\equiv}C-COONa$	3.41	34 920	$3.51.10^{14}$	+5.6
$(CH_3)_3C-C{\equiv}C-COONa$	3.36	33 870	$8.03.10^{13}$	+2.7
$HC{\equiv}C-COOH$	67.2	29 850	$6.12.10^{12}$	−2.5
$CH_3-C{\equiv}C-COOH$	2.09	31 190	$1.21.10^{12}$	−5.7
$(CH_3)_3C-C{\equiv}C-COOH$	2.30	29 850	$2.12.10^{11}$	−9.1

* Calculated by the Arrhenius equation.

of the salts of propiolic and tetrolic acids is primarily due to a difference in the activation energies, while that between the rates of the salts of propiolic and *tert.*-butylpropiolic acids can be attributed to a difference in the value of the frequency factor. The relationships between the acids are approximately similar to those found for the salts. The difference in the rates of *tert.*-butylpropiolic and propiolic acid is determined primarily by the (+ I) inductive effect of the *tert.*-butyl group. If the rate of decomposition of sodium tetrolate were solely determined by the inductive effect of the methyl group, one would expect sodium tetrolate to decompose with approximately the same rate as sodium propiolate, and also the other kinetic quantities should have approximately the same values for these two salts, since the inductive effect of the methyl group is small compared to that of the *tert.*-butyl group. The fact that sodium tetrolate decomposes 25 times slower than sodium propiolate and at approximately the same rate as sodium *tert.*-butylpropiolate can readily be explained by the hyperconjugation in sodium tetrolate in the initial state (see below). As in methylacetylene, π_y- and π_z-hyperconjugation can be postulated in sodium tetrolate. This implies a lower energy of the compound



initial state transition state

in the initial state. As the decomposing molecule enters the transition state, a partial negative charge develops at one end of the triple bond (transition state above). This charge, whose separation is opposed by the hyperconjugation, counteracts the hyperconjugative transfer of electrons and consequently hyperconjugation does not exist in the transition state. A similar explanation has been proposed by Ingold [6] for the differences observed between the dipole moments of *iso*cyanides and those of nitromethane and nitrobenzene, in which both conjugation and hyperconjugation are possible.

The difference, 1 310 cal., between the activation energies of sodium propiolate and sodium tetrolate is not as large as the energy of hyperconjugation for sodium tetrolate owing to solvation effects in aqueous solution.

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The Energy of the Methyl Group-Triple Bond Hyperconjugation

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It has been qualitatively shown previously that the difference in the rates of decarboxylation of sodium tetrolate and sodium propiolate is mainly determined by hyperconjugation. In the present paper a quantitative study of the phenomenon is presented which is based on the assumption that under ideal conditions the difference in the activation energies for the decarboxylation of these compounds is equal to the experimentally determined energy of hyperconjugation between a methyl group and the triple bond. By determining the rates of decomposition of the above salts under conditions where solvation is a minimum, a limiting value, 3 440 ± 200 cal., has been calculated for the activation energy difference. By adding 1 100 cal. to this value as proposed by R. S. Mulliken, the value of the theoretical energy of hyperconjugation comes out to 4 540 cal., which is practically the same as the value computed by R. S. Mulliken and coworkers for the energy of hyperconjugation in methylacetylene by the molecular orbital method.

In a previous work [1] it was concluded the difference in the rates of decomposition of sodium propiolate and sodium tetrolate is primarily due to hyperconjugation in the latter compound. In the present study, this conclusion is considered on the basis of additional kinetic data.

EXPERIMENTAL

Materials. The propiolic, tetrolic and *tert.* butylpropiolic acids employed were those used in the previous studies [1,2]. The sodium salts of these acids were prepared as described earlier [2]. Dioxan (Merck) was purified by the method of Hess and Frahm [3], after which its melting point was 11.8° C. Ethanol (absolute ethanol, Oy Alkoholiliike, Rajamäki) was dried with aluminium using $HgCl_2$ as activator. Benzene (Merck, benzene free from tiophene) was refluxed over sodium wire and then distilled. The water was ordinary distilled water.

Measurements. The kinetic measurements were performed as described previously [2] using the same calibrated apparatus.

RESULTS

The decomposition of sodium propiolate was studied in an ethanol-dioxan mixture, that of sodium tetrolate in absolute ethanol, in two ethanol-benzene mixtures and in a dioxan-water mixture, and the decomposition of sodium *tert.* butylpropiolate in a dioxan-water mixture. In all experiments the initial concentration of the salt was 0.02 *M*. In the case of the above solvent mixtures, an attempt was made to increase the proportion of the non-polar component as far as possible without exceeding the precipitation limit of the salt at room temperature. This did not succeed for sodium tetrolate and sodium *tert.* butylpropiolate in the case of the dioxan-water mixtures, since these salts give 0.02 *M* solutions even in the 95 % dioxan-water mixture, which latter mixture, however, appeared to be unstable at the temperatures at which the experiments were performed. For this reason, sodium tetrolate was studied only in the approximately 80 % dioxan-water mixture and sodium *tert.* butylpropiolate in the approximately 60 % dioxan-water mixture. All the reactions were conducted in a nitrogen atmosphere.

The decompositions followed the first-order law in all solvents studied and the maximum deviations of the log k—$1/T$ points from the Arrhenius plots did not exceed 1 % except for sodium *tert.* butylpropiolate, for which the maximum deviation was approximately 2 %. The calculations were carried out using the same equations as previously [2].

The rate constants are given in Table 1 and the values of the derived kinetic quantities in Table 2. The activation energies E and frequency factors A have been calculated by the method of least squares. The values of the free energy of activation ΔG^*, heat of activation ΔH^* and the entropy of activation ΔS^* refer to 100° C.

Table 1. Specific reaction rates for sodium propiolate, sodium tetrolate and sodium tert. *butylpropiolate in various solvent mixtures.*

Reactant	Solvent composition in wt %		10^6 k sec.$^{-1}$				
			73.85°	82.32°	90.64°	98.92°	106.78°
HC≡C—COONa	Dioxan, 28.77% + EtOH		3.71	9.27	25.9	69.1	167
		106.78°	114.88°	122.39°	130.19°	137.33°	144.32°
CH_3—C≡C—COONa	Dioxan, 80.59%+H_2O			29.1	69.2	150	302
CH_3—C≡C—COONa	Ethanol	5.38	*	33.0	**	160	
CH_3—C≡C—COONa	Benzene, 52.59 % + EtOH	4.90		30.9		156	
CH_3—C≡C—COONa	Benzene, 58.90 % + EtOH	4.84	12.8	30.8	73.0	157	
				122.39°	130.19°	137.23°	144.32°
$(CH_3)_3C$—C≡C—COONa	Dioxan, 61.32 %+H_2O			19.1	42.0	87.1	178

* 115.03°: 14.2.
** 130.14°: 76.9.

Table 2. Derived kinetic quantities for the decomposition of sodium propiolate, sodium tetrolate and sodium tert. *butylpropiolate in various solvent mixtures.*

Reactant	Solvent composition in wt %	A sec.$^{-1}$	E cal.	ΔG^*cal.	ΔH^*cal.	ΔS^*E.U.
$HC{\equiv}C{-}COONa$	Dioxan, 28.77 % + EtOH	$3.59 \cdot 10^{14}$	31 860	29 030	31 130	+ 5.6
$CH_3{-}C{\equiv}C{-}COONa$	Dioxan, 80.59 % + H_2O	$7.06 \cdot 10^{14}$	35 080	31 740	34 340	+ 7.0
$CH_3{-}C{\equiv}C{-}COONa$	Ethanol	$3.57 \cdot 10^{14}$	34 460	31 620	33 710	+ 5.6
$CH_3{-}C{\equiv}C{-}COONa$	Benzene, 52.59 % + EtOH	$7.59 \cdot 10^{14}$	35 100	31 700	34 360	+ 7.1
$CH_3{-}C{\equiv}C{-}COONa$	Benzene, 58.90 % + EtOH	$9.60 \cdot 10^{14}$	35 290	31 720	34 550	+ 7.6
$(CH_3)_3C{-}C{\equiv}C{-}COONa$	Dioxan, 61.32 % + H_2O	$5.96 \cdot 10^{13}$	33 490	31 980	32 750	+ 2.1

DISCUSSION

When a molecule decomposes unimolecularly in the gaseous state to give radicals or atoms, the activation energy of the decomposition is equal to the energy of dissociation of the bond that is broken, provided the activation energy of the reverse reaction is zero [4].

As has been proposed previously [1], the initial state of sodium tetrolate is characterized by a π_y- and π_z-hyperconjugation between the methyl group and the triple bond, which decreases the potential energy of sodium tetrolate and at the same time retards its decarboxylation. The hyperconjugation is presumably of the same degree as in methylacetylene, *i. e.* a true hyperconjugation between the methyl group and the triple bond, since evidently conjugation does not occur between the triple bond and the carboxylate ion group in sodium tetrolate [2]. As the decomposing molecule enters the transition state, a partial negative charge develops at one end of the triple bond and prevents hyperconjugation to occur in the tetrolate ion. In order that the tetrolate ion can attain the same transition state structure as the propiolate ion, additional energy corresponding at least to the energy of hyperconjugation between the methyl group and triple bond is required in the case of the former in the gaseous state [1]. This is schematically shown in Fig. 1.

It is impossible to make measurements on the behaviour of ions in the gaseous state or even in non-polar solvents. An attempt has been made to approach the ideal conditions by conducting the decomposition reactions of the salts in aqueous and alcoholic solvents containing such proportions of non-polar components that the limits of precipitation of the salts are almost attained. Measurements on unimolecular reactions conducted in non-polar solvents are comparable with measurements conducted on such reactions in the gaseous state only if the initial state and the transition complex do not greatly differ from each other in structure. This condition appears to be fulfilled by the reactants under study, but since the purpose has been only to determine the activation energy difference, it is sufficient that the structures of the transition states of both the propiolate and tetrolate ions do not differ greatly. This appears to be true since the entropies of activation for the decompositions of these two compounds are only slightly different (Table 2). In fact the value of ΔS^* for sodium tetrolate is greater than that calculated for sodium propiolate,

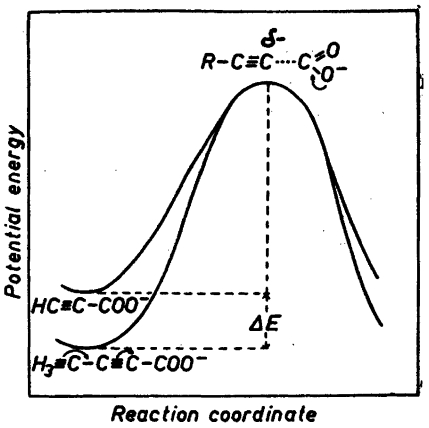

Fig. 1. A schematic diagram showing Potential-Energy-Reaction-Coordinate curves for the decomposition of sodium propiolate and sodium tetrolate. The zero levels for the two compounds do not coincide.

which is explained by the hyperconjugation in the former. According to the view of solvation outlined previously [2], an ideal solvent for these compounds would be one that is unable to release and form hydrogen bonds at different solvation sites on the reacting molecule. On the other hand, observations made during the course of this study which are being studied in more detail, indicate that the solvent cannot be a hydrocarbon owing to the presence of the hydrocarbon chain in the decomposing compounds. Since carbon tetrachloride is unstable to alkalis, dioxan has been chosen as the non-polar solvent, particularly as it was found to conform with the requirements for an ideal solvent.

The variation of the activation energy for the decomposition reactions of sodium propiolate and sodium tetrolate with the composition of the solvent is shown in Fig. 2. It will be seen from the figure that the activation energy for sodium propiolate increases at first as the proportion of the non-polar solvent increases but, later on, decreases fairly steeply. The activation energy for sodium tetrolate, however, remains fairly constant with only a slight gradual rise over the solvent range. In the case of sodium propiolate an ideal limiting

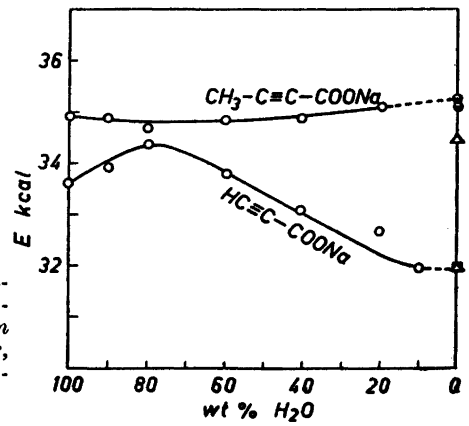

Fig. 2. The variation of the activation energy with solvent composition for the decomposition of propiolate and tetrolate ions in various solvent mixtures. ○ *dioxan-water,* △ *ethanol,* □ *dioxan-ethanol,* ◒ *benzene-ethanol.*

value appears to be attained when the limit of precipitation is approached, since the same value for the activation energy is found for the dioxan-water, ethanol-water and ethanol solutions. In the case of pure ethanol, sodium propiolate dissolves only with difficulty in this solvent to give a 0.02 *M* solution. As already mentioned, this precipitation limit could not be attained for sodium tetrolate in the case of the dioxan-water mixtures. However, the value found for the activation energy near the precipitation limit in the benzene-ethanol mixture appears to be the same as that which would be obtained by extrapolating the activation energy curve plotted for the reaction in the dioxan-water mixtures.

By subtracting the limiting value, 31 850 cal., of the activation energy for sodium propiolate from the corresponding value for sodium tetrolate, 35 290 cal., we obtain 3 440 cal. which is thus assumed to be the value of the hyperconjugation energy between a methyl group and a triple bond. This difference is believed to be accurate to $\pm$ 200 calories. To this experimental value, a further 1 100 cal. must be added as proposed by R. S. Mulliken and co-workers [5] to take into account the fact that work must be done when the acceptor bond decreases and the donor bond increases in length in hyperconjugation against the forces which tend to maintain the normal lengths of the bonds. The resulting value for the theoretical hyperconjugation energy is thus 4 540 cal. Using the molecular orbital method, Mulliken and co-workers [5] have theoretically calculated for the hyperconjugation energy in methylacetylene the value 4 520 cal. and on the basis of heats of combustion have obtained the experimental value 3 600 $\pm$ 200 cal. which by adding the correction 1 100 cal. gives 4 700 cal. as the theoretical value. The accordance between these values and the value found in the present study is very satisfactory.

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Accurate Determination of Thermal Conductivities

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The electrical method for measuring thermal conductivities, introduced by Stålhane and Pyk [1] and Eucken and Englert [2], has been put on a sound basis and the accuracy increased, for both solids and liquids, up to about ± 0.3 %. The principle is that of the classical methods for gases in which the temperature of an electrically heated wire, immersed in the medium, is measured over a suitable time interval. The temperature of the wire is determined continuously by a resistance measurement, not over the whole length of the wire but only over a central section. In this way undesired conduction effects at the ends of the cell and the corrections for them can be neglected. Also, because unsteady state conditions are used, no correction is required for the temperature discontinuity at the wire-medium interface. A number of precautions and arrangements, necessary for accurate work, have been examined in detail, in order to adapt the method for precision routine measurements.

The method can be used for solids, whose melting points are not too high, by pouring the liquified substance directly into the cell, care being taken to see that no gas bubbles settle out on the wire due to incomplete degasification. In the case of liquids, high accuracy is obtained for fluids of sufficiently high viscosity or sufficiently low thermal dilatation, as will be described in a following paper.

THE HOT WIRE PRECISION METHOD

1. General principles

Thermal conductivities, apart from their undoubted industrial importance, can also be used to throw light upon the structure of the substances studied and the mechanism by which heat is transmitted through them. The two main difficulties of this work are, first, to insulate the substance in such a way that heat will flow only in the desired direction and, second, to avoid errors in the temperature measurements due to the temperature jump which exists between the substance under investigation and the temperature measuring device. Accurate methods fall into two groups — those in which uni-dimensional heat conduction is investigated (generally steady state), proper care being taken with the insulation of the substance to ensure correct

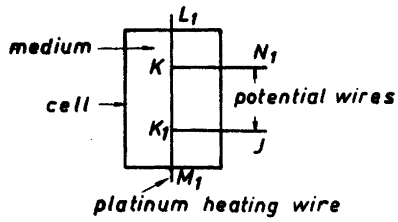

Fig. 1. Principle of the hot wire method.

boundary conditions, and those in which two-dimensional heat conduction is studied (both steady and unsteady state). With the former group and likewise with the steady state methods in the latter group, the insulation and temperature jump errors are likely to be appreciably large. Both of these errors can be avoided by using instead a certain two-dimensional unsteady state method. The principles of this method were first laid down by Stålhane and Pyk [1]. It was first used for accurate measurements by Eucken and Englert [2] but the cell and electrical system used by them were inconvenient and have been considerably simplified in the present work. Heat was provided by passing an electrical current through a thin platinum wire immersed in the medium and situated in the axis of the cylindrical glass containing vessel (Fig. 1).

As soon as current passed through the wire, the temperature of the wire increased but the rate of increase decreased as heat was conducted away by the medium. From a resistance determination on the platinum wire (*i.e.* a temperature determination at the axis) at various times during the passage of the current, the thermal conductivity could be calculated. Moreover by measuring the resistance of the section KK_1 only, using the potential wires shown in the diagram, it was possible to assume strictly two-dimensional heat conductivity and thus there was no necessity for applying a correction for the three-dimensional heat flow at the ends as is usual in methods of this type. Platinum, because of its superior resistance qualities, *e.g.* resistance stability over long time periods and freedom from corrosion, can be considered to be the most suitable material for the wire.

The mechanical design of Eucken and Englert's cell [2] was felt to be unnecessarily complicated and it was thought preferable to construct a simpler cell (Fig. 2) which could be easily filled with liquids and also solids whose melting points were not too high.

The diameter of this simpler cell was 3 cm, the length of the KK_1 section was 10 cm and the distances from the points K and K_1 to the ends of the cell were 4 cm. The lengths of the platinum sections of the potential wires were about 4 cm. Thermo-pure platinum was used for the central wire and chemical pure platinum for the potential wires.

The difficulty of making a neat join between the platinum wires was overcome by the use of a small spot welder. The welding current is rather critical; too small currents will not give sufficient heat for welding whilst too high currents will completely burn away the wires at the welding point. With practice, a strong, right-angle join could be made between the potential wires and the central wire without any bead formation at the point of union. The four ends of the platinum wire system were then each sealed into short copper wires, using a small reducing gas-oxygen flame and pushing the platinum wire into the molten

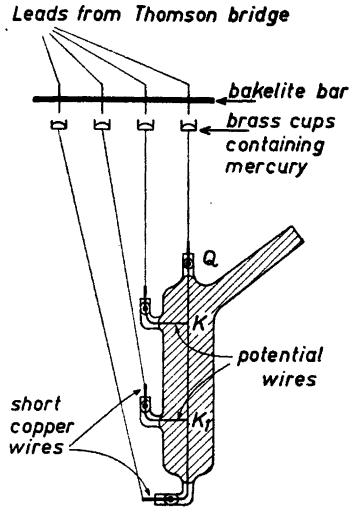

Fig. 2. Pyrex cell designed for the method.

copper bead. The wires were carefully moved into the cell by attaching cotton threads to the copper wires and pulling each section into the correct side arm. Both the copper and the platinum wires were sealed into the side arms — a small cavity containing the copper-platinum join being left unsealed. Thus the cell was made vacuum tight and the platinum wire system protected from mechanical breakage. The final operation was to straighten the central platinum wire in order to get a true cylindrical heat flow during the experiment. This was accomplished by inverting the cell and attaching a 50 g weight to the lower end of the side arm Q (*cf.* Fig. 2) and heating very gently in the middle of this side arm. As the glass began to melt, the weight brought the platinum wire straight. To each of the four short copper wires were soldered long copper wires to the other ends of which were soldered brass cups containing mercury. The cell was connected into the bridge by lowering the bakelite bar. This convenient method also gave freedom from variable contact potentials and resistances at the connection points. The cell was placed in a thermostat bath ($\pm$ 0.005° C) which was filled with transformer oil in order to insulate the cell leads.

With solids, the filling of the cells required care because of the ability of solid organic compounds to dissolve large amounts of air — both in the solid and fluid states. Moreover the difficulty is that many of them dissolve more air in the liquid state and thus on solidification air bubbles are formed in the solid which tend to become attached to the central platinum wire and thus alter completely the heat flow properties of the medium. To overcome this, the solid was alternately melted and frozen several times under vacuum in a large container until all the air bubbles had escaped — it was then melted again and poured carefully into the cell.

2. Electrical circuit

A Kelvin bridge was used to determine the resistance of the KK_1 section and thus the temperature of this platinum wire section (Fig. 3). The Kelvin bridge is usually used for measuring constant resistances and therefore, since the resistance of the platinum wire is continuously changing when the heating

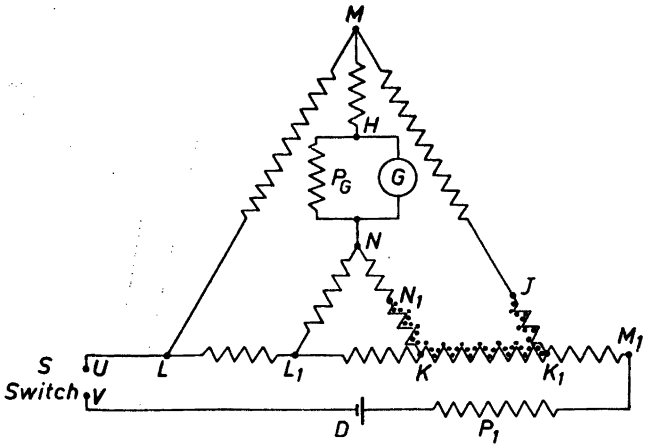

Fig. 3. Kelvin bridge used for the resistance measurements. G: galvanometer. The resistances of the platinum wire sections situated inside the cell are represented by L_1K, KK_1, K_1M_1, N_1K and JK_1 (cf. Figs. 1 and 2).

current is passing through it, the bridge method must be modified in order to determine a continuously varying resistance.

The battery D was used to supply current to the bridge and also to pass the heating current through the axial platinum wire during the experiment. The section KK_1 was that part of the platinum wire which was used to measure the temperature at the centre of the conductivity cell whilst N_1K and JK_1 correspond to the potential wires which were connected to the end points of this section. The two end sections of the central platinum wire are included in the parts of the bridge denoted by L_1K and K_1M_1.

It is necessary to make the central wire as thin as possible to make the heating effect large. Thus the resistance change will be large and hence an increase in accuracy will result. From the practical point of view, *e.g.* mechanical strength and possibility of manipulating the wire *etc.*, 0.1 mm diameter platinum wire was the thinnest possible to use. It was necessary that the potential wires should also be thin so that there would be a negligible disturbance of the two-dimensional heat flow from the central wire. The practical limit to the diameter was about 0.05 mm and it was proved that the disturbance of the heat flow from the axial wire into the medium was small with such a diameter. Because the potential wires were so thin, it was necessary to ensure that only small currents were passed through them. For this reason, ML consisted of a fixed resistance of 100 ohm whilst NK was made up of a fixed resistance of 50 ohm (NN_1) in series with the upper platinum potential wire (N_1K) in Fig. 1. Because the conditions for bridge balancing are:

$$\frac{MK_1}{ML} = \frac{NK}{NL_1} = \frac{KK_1}{LL_1}$$

and $KK_1/LL_1 = 1.25$ (LL_1 consisted of a fixed resistance of 1 ohm), it is seen that NL_1 will be about 40 ohm and MK_1 about 125 ohm. This also guaranteed that the maximum possible changes in the resistances of the potential wires during the experiment due to the temperature alterations were not sufficient to disturb the balance of the bridge. A Leeds and Northrup potentiometer was set up so that the potential differences across LL_1 and KK_1 could be measured. Using these potential differences, the values of the heating current and the KK_1 resistance at the start of the experiment (both required for the calculation of the thermal conductivity) could be computed.

Since the bridge galvanometer deflection can vary continuously, this gives the idea for the measurement of a resistance varying with time. Assuming first that the bridge has been balanced, then, if one of the resistances, say KK_1, is altered, the galvanometer will be deflected from its zero position and for each value of the resistance there will be a corresponding value of the galvanometer scale reading. Thus from a calibration curve of the galvanometer scale against the cell resistance KK_1, the resistance and thus the temperature of the central platinum wire can be determined during the whole of the experiment. However, it would be clearly impossible to use the cell resistance KK_1 for this calibration because of its variation with the passage of current and therefore it was necessary to use instead a continuously adjustable resistance whose value was independent of the current and could also be altered by previously determined small steps. This resistance will be referred to as the "calibration resistance". It is also clear that the initial value of this calibration resistance should be equal to the cell resistance KK_1 (at $t = 0$, *i.e.* before it is altered by the passage of current) and that, for the calibration using the calibration resistance to correspond to the cell resistance, it is necessary that the other elements in the bridge circuit should be the same in both cases. Now ML, NL_1 and LL_1 can be kept the same but this is not possible for MK_1 and NK since parts of these resistances lie within the cell itself (*i.e.* the two potential wires form part of MK_1 and NK). This difficulty can be overcome by placing switches in the circuit so that the parts N_1K, KK_1 and JK_1 (*i.e.* the cell resistance and its potential wires) can be replaced by the calibration resistance and new N_1K, JK_1 resistances (represented by the dotted lines, see Fig. 3). If this new system is balanced by adjusting the new N_1K and JK_1 resistances and using the same values for ML, NL_1, MJ and LL_1 as before, then, because of the balance conditions, the values obtained for the new N_1K and JK_1 will be identical with those obtained previously for N_1K and JK_1 when the cell resistances were contained in the circuit. Thus the bridge circuit containing the calibration resistance can be made identical with that containing the cell and therefore the calibration curve of KK_1 resistance against scale reading, obtained with the calibration resistance, will also apply for the cell.

3. Bridge adjustments performed during an actual experiment

When current flows through the central platinum wire, its temperature rises as a logarithmic function of the time. Thus its resistance rises logarithmically and hence also the galvanometer scale deflection (Fig. 4).

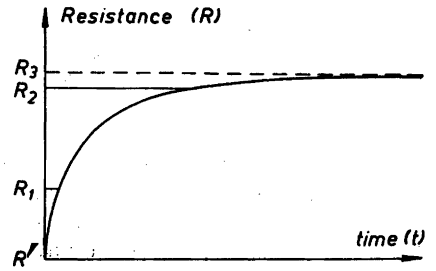

Fig. 4. Resistance versus time for a wire surrounded by a conducting medium and heated by a constant electric current.

The time taken for the resistance increase from the initial KK_1 value, R', to the value R_1, is very short and the accuracy of the measurements in this region is small because of both the rapid resistance change and the lack of accuracy of the time determinations. The region from R_2 to R_3 is useless because the resistance change is too small to permit accurate determinations. Thus the middle region from R_1 to R_2 is the most suitable one for the measurements. This immediately suggests the possibility of altering MJ and NL_1 (see Fig. 3) so that the bridge is balanced when KK_1 is placed at the value R_1 and using such a heating current that the maximum galvanometer scale deflection is reached when the KK_1 resistance has increased to the value R_2. In this way the galvanometer's sensitivity can be used to the greatest advantage. For the experimental currents necessitated by the sensitivity of the galvanometer used here, the resistance R_1 was reached after about 4 sec.

A. In practice the value R_1 was found by trial and error (and MJ and NL_1 were altered so as to obtain balance). One outcome of this procedure was that the galvanometer circuit should not be closed during the experiment until 4 sec after the heating current has been switched on — this was to avoid galvanometer scale deflections in the negative direction which, because of the galvanometer hysteresis, would make inapplicable the previous calibration of galvanometer scale *versus* KK_1 resistance.

B. A similar procedure was adopted when the calibration resistance and its potential leads were placed in the circuit in order to determine the calibration curve — the KK_1 value for the calibration resistance was made equal to R_1 and the same values for MJ and NL_1 were used as were found in *A*. In this way, (1) the calibration curve started from the same KK_1 value as that from which the measurements were begun, (2) the other elements in the bridge, *e.g.* MJ, JK_1, NK, ML, LL_1, NL_1 were the same during the actual experiment with the cell and during the calibration, and thus (3) the calibration curve of galvanometer scale reading *versus* KK_1 resistance was applicable to the scale deflections obtained during the experiment.

An optical system was designed so that the galvanometer scale and a chronometer could be photographed simultaneously. Thus it was possible to calculate the resistance of the central platinum wire during the experiment as a function of time. The order in which the bridge adjustments were carried out in practice is summarized as follows:

1) The cell was connected into the bridge (*cf.* Fig. 3) and MJ and NL_1 were adjusted so that balance was obtained.

2) The calibration resistance was connected into the bridge and its KK_1 section was made equal to the KK_1 section of the cell resistances using the Leeds and Northrup potentiometer mentioned in section 2.

3) With the calibration resistance in the bridge, N_1K and JK_1 were adjusted so that balance was obtained.

4) With the cell in the bridge, the value of R_1 was found by trial and error (and MJ and NL_1 were altered to obtain balance, see *A.* above) after choosing a heating current such that the maximum galvanometer deflection was reached when the KK_1 cell resistance had increased to the value R_2.

5) The calibration resistance was connected into the bridge and its KK_1 section was made equal to the value R_1 found in (4). Using the values for MJ,

NL_1 and the heating current found in (4), the calibration curve was obtained by altering KK_1 stepwise from R_1 to R_2, photographing the scale after each alteration and plotting the scale values obtained against the KK_1 resistance.

6) The cell was then connected in and the heating current switched on. The galvanometer scale and the chronometer were photographed simultaneously about 20 times during the passage of current. Using the calibration curve, the resistance corresponding to each galvanometer scale reading could be obtained. Thus the resistance (and hence the temperature) of the central platinum wire could be calculated as a function of the time during the whole of the experiment.

In (1), (2), (3), (4) the potential placed across the bridge was chosen (using the resistance P_1, Fig. 3) sufficiently large to make the adjustment accurate enough but sufficiently small so that the resistances in the bridge were not appreciably altered.

Great care had to be taken with the design of the switches used to replace the cell resistances by the calibration resistance and its potential leads. All common types of switches were found to give much trouble due to their contact potentials and resistances. Because of the variability of these contact phenomena, it was difficult to maintain the balance of the bridge over long periods of time. All of the switches were therefore specially designed so that contact was made and broken between an amalgamated copper rod and mercury — the contact resistance and potential being negligible in that case.

Now the calibration curve was obtained when the galvanometer was stationary. Therefore a correction must be applied to each scale reading determined during the experiment since the galvanometer is then in motion and possesses a finite oscillation period. Moreover since the galvanometer speed varies during the experiment, the correction will also vary from point to point on the scale. However, instead of correcting each scale value, it is shown in the next section that a great simplification results if the correction is applied to the time values.

4. Galvanometer theory

The equation of motion for the galvanometer is

$$K_0 \frac{d^2\Phi}{dt^2} + \left(p_0 + \frac{q^2}{R_T}\right) \frac{d\Phi}{dt} + D\Phi = q\ I(t) \qquad (1)$$

where

Φ = angle of deflection of the mirror
= 1/2 (angle of deflection of the light beam)
K_0 = moment of inertia of the rotating system
$-p_0 \frac{d\Phi}{dt}$ = moment on the mirror due to air resistance
q = the dynamical galvanometer constant
R_T = total resistance of the galvanometer circuit
$-D\Phi$ = elastic torque
$I(t)$ = current through the galvanometer (a function of time).

The constants in this equation were calculated from experimental determinations of the following experimentally observable galvanometer properties:

statical galvanometer constant, oscillation period, logarithmic decrement and critical damping resistance.

Instead of the independent variable Φ, it is more convenient to use the deflection, x, on the galvanometer scale where $x = 10^2 \cdot 2\Phi$ (x is in cm). The equation of motion was found to be

$$2.44 \cdot 10^{-3} \frac{d^2x}{dt^2} + \left(1.77 \cdot 10^{-3} + \frac{10.3}{R_T}\right) \frac{dx}{dt} + x = 0.96 \cdot 10^7 \; I(t) = x_0(t) \quad (2)$$

where $x = x(t)$, a function of time, is obtained from the experimental film, and thus using equation (2) the value of $x_0(t)$ can be found. Hence every point of the experimentally found, $x(t)$ *versus* time, curve must be corrected using this equation. To avoid the time consuming numerical work, a zero time correction was tried instead. Equation (2) can be written as

$$x_0(t) = x + P \frac{dx}{dt} + Q \frac{d^2x}{dt^2} \quad (3)$$

Now the right hand side represents the first three terms in a Taylor expansion if $Q = 1/2\, P^2$. This condition can be satisfied since the value of P can be altered by changing R_T. If the terms in the Taylor expansion after the first three are neglected, equation (3) can be written

$$x_0(t) = x(t + P) \quad (4)$$

Thus instead of correcting all of the x values determined experimentally, it is only necessary to subtract the constant term P from all of the time values. Hence the galvanometer correction can be expressed by adding a positive term to the zero time. The resistance value, R_T, to make this simple correction possible is obtained from $Q = 1/2\, P^2$, which gives $R_T = 150$ ohm. Therefore, by arranging that the *total* resistance in the galvanometer circuit should have this value, the correction to be applied because of the finite oscillation time of the galvanometer can be expressed as a correction, P, in the zero time where $P = \sqrt{2\,Q} = 0.07$ sec. It can also be shown that the remainder term neglected above is negligible.

It is the sensitivity of the galvanometer which mainly determines the minimum heating current which can be used. Using the minimum current for the substances so far studied, it was found that the time required for the KK_1 resistance to increase from R' to R_2 (*i.e.* the experimental time length) was in all cases about 60 sec. It is of course possible to use higher currents by making a compensatory increase in the galvanometer series resistance MH, *cf.* Fig. 3 (and placing a certain resistance P_G in parallel with the galvanometer so that the condition, $Q = 1/2\, P^2$, is still valid). Moreover in practice slightly greater currents than the minimum were used for reasons of experimental convenience. By suitably increasing the galvanometer series resistance it is possible to obtain the same scale deflection as when the minimum current is used and in addition it results that the experimental time length will also be about 60 sec. However, if the current becomes too large, it may exceed the safe heating limit for some of the resistance elements in the bridge and also, in the case of liquids, it may be large enough to produce convection. In order that

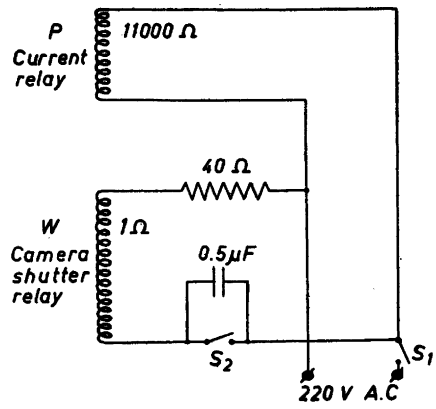

Fig. 5. Relay system for the simultaneous operation of the bridge current switch and the camera shutter.

the assumption of an infinite two dimensional medium should be valid, it is necessary to make the diameter of the cell greater than a certain minimum value. Using the experimental time length of 60 sec, a diameter of 3 cm was found to be suitable for the substances so far studied.

5. Zero time determination

The time determination was obtained by photographing a chronometer. To determine the absolute value t, the zero time when current first began passing through the cell must also be determined. To do this the relay P which switched on the current in the bridge circuit was connected in parallel with the relay W which operated the camera shutter. The circuit is shown in Fig. 5.

Passage of current through the electromagnetic relay P caused contact to be made between two mercury pools, one of which was in contact with the lead U and the other with the lead V (see Fig. 3). This type of relay was ideal for high precision electrical work because no contamination of the surfaces, by which contact was made and broken, took place. When current passed through the relay W, the camera shutter release was depressed and a photograph taken. The experiment was started thus: First switch S_2 was closed and then S_1 thrown. Both relays were therefore activated, *i.e.* the bridge current switched on and a photograph of the chronometer taken simultaneously. Switch S_2 was then opened and afterwards closed every time it was desired to photograph the galvanometer scale to obtain the scale reading. The zero time was the time shown by the chronometer in the first photograph.

However, the zero time must be corrected for the small time difference between the relays, *e.g.* the first photograph was actually taken 0.02 sec before the bridge current was switched on. This value remained constant to within $\pm$ 0.005 sec over a six months' period of daily operation. The time difference was determined using a Cossar double beam oscilloscope and placing a photomultiplier inside the camera. The pulse obtained from the photomultiplier, when the camera shutter opened, deflected one of the oscilloscope beams vertically whilst the passage of current through the switch S (Fig. 3),

when it was closed, was used to deflect the other beam vertically. A horizontal pulse was applied to both beams simultaneously during the interval when the two vertical pulses were registered. Thus by comparing the resulting wave trace with a sinusoidal time base curve, the time difference between the two operations could be determined.

6. Theory of the hot wire method

The two-dimensional solution of the heat conductivity equation, given by Eucken and Englert, assuming an infinite medium and an infinitely thin heating wire is

$$\lambda = \frac{Q}{4\pi} \cdot \frac{\Delta \ln t}{\Delta T} \tag{5}$$

(λ = thermal conductivity of the medium, t = time,
Q = heat liberated by the heating wire in cal/cm/sec,
T = temperature of the wire.)

Ideally, Q should remain constant during the experiment. However, in practice, the resistance of the heating wire alters due to its temperature change and thus Q actually varies with time. The necessary correction to eq. (5) was calculated as follows:

If Q is constant, then the temperature $T(t)$ at a certain point in the medium is

$$T(t) = Q\ f(t) \tag{6}$$

where $f(t)$ is a function of t.

However, if Q is dependent on t (and therefore written $Q(t)$), then the temperature $T_0(t)$, at the point under consideration, will be given by

$$T_0(t) = \frac{\delta}{\delta t} \int_0^t Q(\tau) f(t-\tau) d\tau \tag{7}$$

(this is known as Duhamel's integral [3]).

The battery potential is denoted by E. This voltage is put across the central platinum wire (with resistance R_J at $t=0$, this is the resistance over the *whole* length L_1M_1, *cf.* Fig. 1) and, in series with it, a constant resistance R_K (equal to the resistance P_1 plus the resistance LL_1, *cf.* Fig. 3). Then, if $T_0(t)$ is the temperature at the boundary surface between the wire and the medium, the variable amount of heat liberated during the experiment $Q(t)$ is given by

$$Q(t) = \frac{0.2389}{l} \cdot R'(1 + \alpha\ T_0(t)) \cdot \left[\frac{E}{R_K + R_J(1 + \alpha\ T_0(t))}\right]^2 \tag{8}$$

where R' ohm is the resistance of the KK_1 section of the wire at $t = 0$, l cm is the length of this section and α the temperature coefficient of this resistance. Equation (8) can be written

$$Q(t) = \frac{0.2389\ R'}{l} \left(\frac{E}{R_K + R_J}\right)^2 \cdot (1 + \alpha\ T_0(t))\ (1 + \frac{\alpha R_J}{R_K + R_J}\ T_0(t))^{-2}$$

which, remembering that $\alpha T_0(t) < 0.01$, becomes

$$Q(t) = \frac{0.2389\ R'}{l}\left(\frac{E}{R_K + R_J}\right)^2 (1 + \frac{R_K - R_J}{R_K + R_J} \cdot \alpha \cdot T_0(t)) \qquad (9)$$

We now assume that the value $Q = \frac{0.2389\ R'}{l}\left(\frac{E}{R_K + R_J}\right)^2$, *i. e.* the heat liberated at the start of the experiment, is the one which is to be used in equation (5) for the calculation of λ. Now the wire temperatures $T(t)$, which would be obtained with this ideal constant heat generation Q, can be calculated by applying a correction to the actual wire temperatures $T_0(t)$ obtained during the experiment, as is seen in the following:

Putting $\eta = \frac{R_K - R_J}{R_K + R_J}$, equation (9) becomes

$$Q(t) = Q(1 + \alpha\eta\ T_0(t))$$

Placing this value for $Q(t)$ in equation (7), we get the following value for the temperature of the wire

$$T_0(t) = \frac{\delta}{\delta t}\int_0^t [Q + \alpha\eta\ Q\ T_0(\tau)] f(t-\tau)\ \mathrm{d}\tau$$

which, using eq (6), gives

$$T_0(t) = T(t) + \alpha\eta\ \frac{\delta}{\delta t}\int_0^t T_0(\tau)\ T(t-\tau)\ \mathrm{d}\tau \qquad (10)$$

The second term on the right hand side is only a small correction and hence in it $T_0(\tau)$ can be replaced by $T(\tau)$. For small values of $r^2/4at$ it can be shown that

$$\frac{\delta^2}{\delta t^2}\int_0^t T(\tau)\ T(t-\tau)\ \mathrm{d}\tau = \frac{\delta}{\delta t}[T(t)]^2 \qquad (11)$$

which gives

$$\frac{\delta}{\delta t}\int_0^t T(\tau)\ T(t-\tau)\ \mathrm{d}\tau = [T(t)]^2 - \text{constant} \qquad (12)$$

Equation (10) then becomes

$$T(t) = T_0(t) - \alpha\ \eta\ [T(t)]^2 + \text{constant} \qquad (13)$$

Now, equation (13) shows that the correction is nonlinear and thus ought to be made separately for each experimental point used for the determination of the thermal conductivity. However, in the temperature range used in the present experiments, the correction term is small and does not change greatly. Thus the correction can be made more simply as follows:

From equation (13), using the delta symbol for the changes in the variables and dropping the notation for the independent variable t, we have

$$\Delta T = \Delta T_0 - \alpha\eta\Delta T^2 \simeq \Delta T_0 - \alpha\eta\Delta T_0^2$$

Now if T_1 is the temperature of the central platinum wire when resistance measurements are begun and if T_2 is the temperature at the end of the resistance

measurements (*i.e.* T_1 and T_2 correspond respectively to R_1 and R_2 which are the end points of the experimental resistance range in Fig. 4) then

$$\frac{\Delta T}{\Delta T_0} = 1-\alpha\eta \frac{\Delta T_0^2}{\Delta T_0} = 1-\alpha\eta \left(\frac{T_2^2-T_1^2}{T_2-T_1}\right) = 1-\alpha\eta\ (T_2 + T_1)$$

Thus $\frac{\Delta T}{\Delta T_0} = 1-2\ \alpha\eta T_c$ (14) where T_c is the mean temperature. For the experimental evaluation of this correction factor it is more convenient if T_c is expressed in terms of resistances which can be measured during the experiment. Now $\alpha T_c = \frac{1}{R'}\ [R'(1 + \alpha T_c)-R']$.

If the resistance of the central platinum wire is R_c when its temperature is T_c then $R_c = R'(1 + \alpha T_c)$.

Therefore $\alpha T_c = \frac{1}{R'}\ [R_c-R']$

and $$\frac{\Delta T}{\Delta T_0} = \frac{R'-2\eta(R_c-R')}{R'} \qquad (15)$$

This correction equation is used as follows: In the experiment $\frac{\Delta \ln t}{\Delta T_0}$ is actually measured but, according to equation (5), $\frac{\Delta \ln t}{\Delta T}$ is required. Equation (5) can be written:

$$\lambda = \frac{Q}{4\pi} \cdot \frac{\Delta \ln t}{\Delta T_0} \cdot \frac{\Delta T_0}{\Delta T}$$

and using equation (15)

$$\lambda = \frac{Q}{4\pi} \cdot \frac{\Delta \ln t}{\Delta T_0} \cdot \frac{R'}{R'-2\eta(R_c-R')} \qquad (16)$$

If I amp is the current through the central platinum wire at $t = 0$ and R ohm the resistance of the KK_1 section of this wire during the experiment, then

$$\lambda = \frac{R'I^2}{4\pi l} \cdot 0.2389 \cdot \alpha\ R' \cdot \frac{\Delta \ln t}{\Delta x} \cdot \frac{\Delta x}{\Delta R} \cdot \frac{R'}{[R'-2\eta(R_c-R')]} \qquad (17)$$

Using equation (17), it is seen that the thermal conductivity, λ, can be calculated from determinations of the slope of the calibration curve, $\frac{\Delta x}{\Delta R}$, and the slope of the experimentally-determined galvanometer scale reading *versus* ln time curve, $\frac{\Delta \ln t}{\Delta x}$. All of the other quantities in this equation are constants and can be obtained from direct potentiometric measurements on the particular cell used.

7. Correction for the differing heat capacities of the heating wire and of the medium

In the theoretical case, heat is generated in a mathematical line and then flows out into the medium. However, in the method used here, heat is generated in a thin cylinder of radius 0.005 cm. In order to apply the theory of the

theoretical case, it is therefore necessary to assume that, if the heat generated is the same, the heat flow in the medium for radial distances $r \geqslant 0.005$ cm is the same in both cases (assumption A).

Now, in the theoretical case, the medium situated in the region $r < 0.005$ cm will take up a certain amount of heat depending on its heat capacity. However, this amount of heat will be different to that taken up by the platinum wire in the actual case because of the wire's different heat capacity. The correction to be applied will now be estimated.

If we assume a constant amount of heat Q cal/cm/sec generated in the platinum wire, then the amount $c_1\gamma_1\pi r_2^2\ T_0'(t)$ is taken up by the wire and the rest $Q_1(t)$ streams out from the wire into the medium, where

$$Q_1(t) = Q - c_1\ \gamma_1\pi\ r_2^2\ T_0'(t) \tag{18}$$

(c_1 = specific heat of platinum, γ_1 = density of platinum, r_2 = radius of the wire (0.005 cm), $T_0(t)$ = temperature of the wire and $T_0'(t) = \frac{\delta}{\delta t}\ [T_0(t)]$).

The platinum wire is now replaced by a hypothetical wire of the same specific heat (c) and density (γ) as the medium. If we again assume a constant heat supply, Q, then the part streaming out from the wire is, to a first approximation,

$$Q_2(t) = Q - c\gamma\pi r_2^2 T_0'(t) \tag{19}$$

Using this value for $Q_2(t)$ and the notation of Duhamel's integral (equation (7)), we find that the temperature of this hypothetical wire, and hence the temperature $T(t)$ at $r = 0.005$ cm in the medium in the theoretical case (this follows from assumption A above), is given by an expression

$$T(t) = \frac{\delta}{\delta t}\int_0^t [Q\ -\ c\gamma\pi r_2^2 T_0'(\tau)]\ f(t-\tau)\ \mathrm{d}\tau \tag{20}$$

Substituting the value for Q from equation (18), we get

$$T(t) = \frac{\delta}{\delta t}\int_0^t Q_1(\tau)\ f(t-\tau)\mathrm{d}\tau + \frac{\delta}{\delta t}\int_0^t (c_1\gamma_1 - c\gamma)\pi r_2^2\ T_0'(\tau)\ f(t-\tau)\ \mathrm{d}\tau \tag{21}$$

Now the first term on the R.H.S. is the temperature $T_0(t)$ of the platinum wire and hence, putting $\beta = (c_1\gamma_1 - c\gamma)\ \frac{\pi r_2^2}{Q}$ and neglecting second order terms, we can write

$$T(t) = T_0(t) + \beta\ \frac{\delta^2}{\delta t^2}\int_0^t T_0(\tau)\ T_0(t-\tau)\ \mathrm{d}\tau \tag{22}$$

Using equation (11), we find

$$T(t) = T_0(t) + \beta\ \frac{\delta}{\delta t}\ [T_0(t)]^2$$

$$i.e.\ T(t) = T_0(t) + 2\beta\ T_0(t)\ T_0'(t) \tag{23}$$

This can be written as a Taylor expansion

$$T(t) = T_0(t + 2\beta\ T_0(t)) \tag{24}$$

since the remainder term which has been neglected is negligible. Thus the correction for the differing heat capacities of the wire and the medium can be expressed by adding a correction term τ_3 to the zero time where

$$\tau_3 = 2\beta\ T_0(t) \tag{25}$$

The numerical evaluation of τ_3 can be performed sufficiently accurately as follows:

For ideal two-dimensional heat flow with a constant amount, Q, of heat liberated at a mathematical line in an infinite medium, the temperature at a point r cm from the line is given by

$$T(t) = \frac{Q}{4\pi\lambda} \int_0^t (e^{-r^2/4a\tau}/\tau)d\tau \tag{26}$$

where $a = \frac{\lambda}{c\gamma}$.

For small values of $r^2/4at$, *i. e.* for relatively large time values, equation (26) becomes

$$T(t) = \frac{Q}{4\pi\lambda} (\ln t + \ln \frac{4a}{r^2} - \gamma') \tag{27}$$

where γ' is Euler's constant (0.5772).

Now $T_0(t)$, the temperature of the wire, is given sufficiently accurately by equation (27) on putting $r = r_2$. During the experiments log t increased from 0.5 to 1.5 and could be taken equal to 1.0 for the calculation of τ_3 since the resultant error was small. Thus

$$T_0(t) = \frac{Q}{4\pi\lambda} (\ln \frac{4a}{r_2^2} + 1.7) \tag{28}$$

Putting this value for $T_0(t)$ in equation (25), substituting the value for β and noting that $c_1\gamma_1 = 0.64$ for platinum, we get

$$\tau_3 = \frac{r_2^2}{2\lambda} (0.64 - c\gamma) (\ln \frac{4a}{r_2^2} + 1.7) \tag{29}$$

For all solutions studied, τ_3 was found to be small due to the thinness of the heating wire, *e. g.* $\tau_3 = +0.03$ (naphthalene), -0.02 (water), -0.01 (glycerol) sec. This correction was combined with the two other zero time corrections given above, *i. e.*

(1) the time difference between the current and camera shutter relays (p. 649).
(2) the zero time correction due to the inertia of the galvanometer (p. 648).

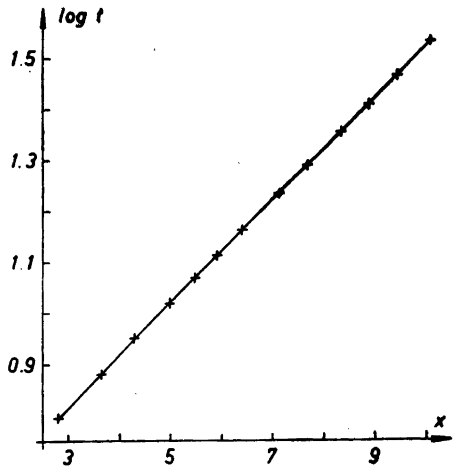

Fig. 6. x (the galvanometer scale reading) as a function of log t for a diphenylmethane/ naphthalene mixture. Temp.: 20.0° C, I = 0.28 amp.

EXPERIMENTAL

In equation (17), a and l were determined for each cell before it was used for experiments while I, R′ and R_c were determined during the experiment using the Leeds and Northrup potentiometer mentioned in section 2. $\frac{\Delta x}{\Delta R}$ was determined from the calibration curve giving the scale reading (x cm) as a function of the KK_1 resistance (R ohm). This curve was almost exactly linear — its mean slope was used for the value of $\frac{\Delta x}{\Delta R}$ and the slight curvature was corrected for by adding small corrections to the x values obtained during the experiment and used for the determination of $\frac{\Delta \ln t}{\Delta x}$.

Initially experiments were carried out on a homogeneous mixture of diphenylmethane and naphthalene containing 2.6 % of the former. Fig. 6 is a plot of x *versus* log t obtained during a typical experiment on this mixture.

The slope of the line was denoted by k. Because it is difficult to see how accurately the line fits the points with such a graph, a simultaneous plot (Fig. 7) of k log $t-x$ *versus* x was always made (with a scale for k log $t-x$ which was 10 times larger than that for x).

By inspection of this latter graph (which should be a horizontal line if the graph of x *versus* log t is linear) it could be seen whether the deviations from linearity fell within the experimental error.

Values obtained from independent measurements for the thermal conductivity, $\lambda \cdot 10^6$, of the mixture of diphenylmethane and naphthalene were: 835, 836, 839, 836, 834, 837, 835, 838, 840, using heating currents from 0.22 amp to 0.28 amp.

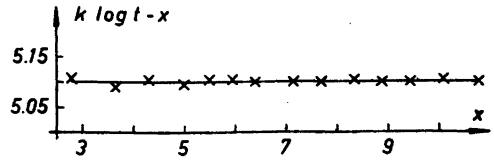

Fig. 7. k log t—x versus x for the same experiment as in Fig. 6. k = 9.92, λ = 0.000835.

The errors in the quantities used in equation (17) were:

$$I^2, \pm 0.1\ \%; \frac{\Delta x}{\Delta R}, \pm 0.3\ \%; \frac{\Delta\ ln\ t}{\Delta x}, \pm 0.1\ \%; l, \pm 0.05\ \%$$

The errors in the resistances used in this equation were negligible and thus the total experimental error was about $\pm$ 0.5 %. Various other errors such as the heat lost from the central heating wire through the potential wires and by radiation, the error resulting from the cross-sectional unevenness of the central wire and others were investigated and found to be negligible. As seen from the above results and also from results on experiments with water and aqueous solutions, at temperatures not too far from the maximum density point, and highly viscous liquids such as glycerine (a following paper), it was possible in practice to reproduce thermal conductivities to within $\pm$ 0.3 %.

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Precision Measurements of the Thermal Conductivities of certain Liquids using the Hot Wire Method

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Experiments have been performed on water, glycerol and aqueous solutions of glycerol, ethyl alcohol and sucrose. In these experiments, no convection was observed during a determination when it was carried out at the temperature of maximum density. But it was found that the further the experimental temperature was from this temperature of maximum density, the earlier the convection started. However, using the experimental readings obtained before convection began, it was possible to calculate the thermal conductivity without any loss of accuracy. Accurate experiments have in this way been performed up to 20° C. The electrically heated wire was always placed vertically so as to minimize convection — it was found that the greater the angle between the wire and the vertical, the sooner the convection began. The method gave an automatic indication of the presence of convection, thus making possible the determination of suitable conditions for precision measurements. The high accuracy, of which this method is capable, is obtainable for highly viscous liquids but is otherwise limited to fluids of low thermal dilatation.

Using the method described in the previous article [1], the thermal conductivities of various liquids and solutions were determined and, in addition, the influence of convection on measurements of this type was investigated. The convection effect can be considered in the following manner. During the first instants of the experiment when convection has not had time to be effective, a straight x *versus* log t curve will be obtained whose slope is determined by the value of the thermal conductivity (and hence the k log t—x *versus* x curve will be a straight horizontal line) where x is the galvanometer scale reading, t the time and k the slope of the x *versus* log t graph (*cf.* Figs. 6 and 7 in Ref.[1]). When convection begins, heat will be lost quicker from the central heating wire because cooler liquid will be moving up to the wire by convection. Thus the thermal conductivity will appear to be greater and the k log t—x graph will curve upwards.

However, if the cell is placed vertical, convection should initially take place in concentric cylindrical surfaces and the convection effect should therefore be

less than in any other case. For example, a typical experiment on a 4.9 % solution of sucrose in water gave the following graph, Fig. 1. The measurement was performed at 4.0° C, near the density maximum of the solution where the thermal dilatation is small.

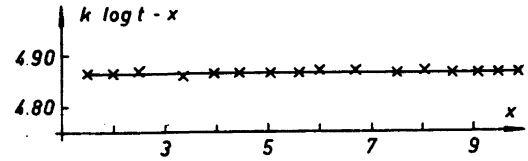

Fig. 1. k log t—x versus x (the galvanometer scale reading) for a 4.9 % aqueous sucrose solution. k = 8.71. Temp: 4.0° C, Cell: placed vertical, I = 0.28 amp (the heating current).

The straightness of the graph indicates the absence of convection. The value calculated for the thermal conductivity was $\lambda = 0.001328$. However, when the cell was placed at an angle of 45° to the vertical, the graph in Fig. 2 was obtained:

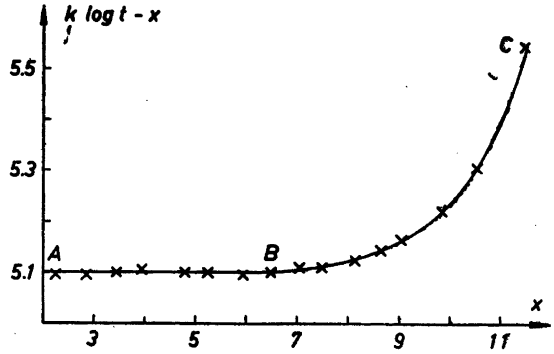

Fig. 2. k log t—x versus x for same solution as in Fig. 1. k = 10.17. Temp: 4.0° C, Cell: 45° to the vertical, I = 0.28 amp, AB: convection—free period, BC: effect of convection.

Convection began at the point *B* after 14 sec. (The total time from *A* to *C* was, for all determinations, about 60 sec.) In an experiment in which the convection effect is greater, the point *B* will occur earlier during the experiment and the deviation of the *BC* section from the horizontal will be larger. However, it should be possible to calculate the thermal conductivity from the initial section, *AB*. A value, $\lambda = 0.001330$, was obtained from the *AB* section in close agreement with the value calculated from Fig. 1. This shows that, if convection did occur in an experiment and a curved k log t—x graph was obtained, it was still possible to calculate an accurate value for the thermal conductivity using the initial, straight portion of the graph. Apart from several

further experiments carried out to check this important conclusion, the cell was always placed vertically so as to minimize convection from this source.

However, there are various other factors which must be taken into account. Firstly, convection will be influenced by the effect of the solute on the viscosity of the solvent. Secondly, convection will be at a minimum at the temperature of maximum density (t.m.d.) and that is why convection was not apparent during the experiments, carried out at 4° C, on the less concentrated aqueous solutions. However, an increase in the concentration results in a decrease of the t.m.d. and should hence give rise to a larger convection effect if the experiment is performed at the t.m.d. of pure water. This explains the occurrence of convection in the experiments on the more concentrated solutions carried out at 4° C.

With glycerol/water mixtures for example, it was found that the convection effect (as judged by the k log t—x graph) increased with increasing glycerol concentration until such a concentration was reached at which the properties of the glycerol became more dominant and that then, because of the high viscosity of solutions containing a high percentage of glycerol, the convection effect decreased as the concentration increased to 100 %. In all cases studied, however, it was possible to calculate the thermal conductivity from the initial, straight portion of the k log t—x graph.

With distilled water at 4° C, no convection was detected both when the cell was vertical and when it was placed at an angle of 45° to the vertical. This shows that the convection arising from a 45° inclined heating wire, under these conditions, is negligible at the t.m.d. However, with water at 20° C and the cell placed vertically, convection began as soon as 20 sec (point E) after the heating current was switched on (Fig. 3).

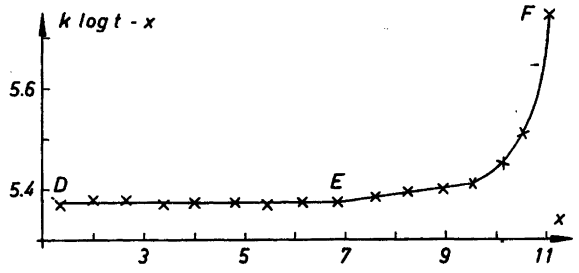

Fig. 3. k log t—x versus x for water. k = 9.47. Temp.: 20.0° C, Cell: placed vertical, I = 0.27 amp. DE: convection-free period, EF: effect of convection.

The thermal conductivity calculated from the DE section was 0.001442.

RESULTS

The measurements are summarized in Table 1. Each value is a mean of up to 8 independent determinations.

Table 1.

Medium	Concentration (weight %)	Current (amp)	Thermal conductivity λ	Convection appeared after	Temp. (° C)
Solution of glycerol in water	4.94	0.28	0.00131_7	20 sec	4.0
	16.00	0.27	0.00123_0	19 sec	4.0
	31.06	0.27	0.00111_0	22 sec	4.0
	63.50	0.28	0.00087_0	—	4.0
	95.24	0.28	0.00069_2	—	4.0
Solution of ethyl alcohol in water	4.0	0.28	0.00129_0	—	4.0
	14.15	0.28	0.00114_9	50 sec	4.0
	28.9	0.28	0.00096_8	20 sec	4.0
Solution of sucrose in water	4.9	0.28	0.00133_0	—	4.0
	15.5	0.28	0.00126_2	—	4.0
	24.75	0.28	0.00119_8	—	4.0
Water		0.27 and 0.32	0.00135_4	—	4.0
»		0.27	0.00144_2	20 sec	20.0
Glycerol		0.27	0.00067_9	—	4.0
»		0.27 and 0.21	0.00067_5	—	20.0

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Bravais' and Kossel-Stranski's Theories of Homopolar Crystals and their Application to Elements

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The two theories are shown to be in fair agreement for simple lattices. The results obtained by the author with the Kossel-Stranski theory differ from those reported by Stranski and co-workers. Both theories conform well with experimental data, as is shown for the crystal habit and structure of a number of common elements, including Cu, Ag, Au, Pb, Pt, Fe, W, V, C, Si, Be, Ti, Mg, Zn, Cd, Se, Te, Sn. In the case of Se and Te serious deviations from the Kossel-Stranski theory have been reported earlier due to inadequate theoretical predictions. The agreement between theory and experiment is best if the outer disturbances are small, as in sublimation, while deviations are found on metal crystals, electrodeposited from complex or molten salts. Electrodeposition as such is not a very disturbing factor.

It is of great interest to relate the habit, *i. e.* the external face development of a crystal to its internal structure. Two main such attempts have been made.

About 1850 Bravais suggested that the order of decreasing importance of the outer surfaces would be the same as the order of decreasing close-packing of the corresponding lattice planes [1]. Hence, this idea has also been referred to as the "reticular density" theory [2]. It has been elaborated in later years, mainly by mineralogists [3–5].

The other theory was developed in 1928 by Kossel [6] and has later been elaborated by Stranski and co-workers [7–9]. This theory considers the number of closest neighbors of a lone particle, settling on a face to begin a new layer. It may therefore be termed the "nearest neighbors' " theory. It was reviewed by several authors [2,10–12].

BRAVAIS' OR RETICULAR DENSITY THEORY

The more closely packed with molecules a set of planes is, the wider is their spacing. It is easily shown that $D = \frac{n}{V} \cdot d$, where D = reticular density (number of molecules per unit area), d = interplanar spacing, V = volume of unit cell, n = number of molecules per unit cell.

The reticular density theory can be formulated in the following way: In close-packed planes the molecules form a dense aggregate and neutralise each other's bonds so that few free valencies are directed towards the outer space. Furthermore, a molecule trying to start a new such close-packed plane will be located relatively far from the molecules of the crystal and will form an excrescence on the surface of the latter. Hence, the new molecule will be but loosely bonded and the tendency for the formation of a seed will be low. On the other hand, a less densely packed plane will consist of molecules less tightly knitted together, which will therefore have more residual valencies pointing out from the crystal. Furthermore, a molecule trying to start a new plane will be close to the molecules of the outermost planes of the crystal and will not project very much above the surface of the crystal. Hence, it will have a greater tendency to stay and form a seed. It should be stressed that the reticular density theory does not take account merely of the outermost plane since a small spacing will mean that the lone molecule is close to the entire crystal.

According to the above, the smaller the reticular density and interplanar spacing of a surface is, the faster will it grow normally to itself. It is obvious, however, that the most rapidly growing surfaces will soon disappear from the crystal and in the final crystal only the surfaces of slowest perpendicular growth will persist. But these are the close-packed surfaces.

Burton and Cabrera [13] and also Hartman and Perdok [14] have divided the faces of a crystal into three different classes, *viz.* close-packed or flat faces, F-faces, containing 2 or more intersecting sets of parallel, close-packed atom chains; stepped or S-faces, containing one set of parallel, close-packed atom chains; and kinked or K-faces, containing no such chains. The directions of close-packed atom chains, called periodic bond chain vectors by Hartman and Perdok [14], correspond to the preferred directions of growth and to important zone directions of the crystal.

It is interesting to note that the order of decreasing importance of the crystal faces according to the reticular density theory will be the same as the sequence in which the first order reflections appear on an X-ray powder recording, proceeding from small to large angles [3]. If the space lattice is known the order of decreasing spacings d can easily be calculated from formulas given in textbooks of X-ray crystallography. The calculation may be checked with the ASTM X-ray data cards.

KOSSEL-STRANSKI'S OR NEAREST NEIGHBORS' THEORY

According to the Kossel-Stranski theory the number of nearest, second-nearest and third-nearest neighbors (and so on) of a lattice particle, put together in an expression $n_1/n_2/n_3...$, are considered to be a measure of the bonding energy of the particle in question. Growth of the crystal will normally proceed by the addition of a molecule to an uncompleted lattice row. This process is called the *wiederholbarer Schritt* (the repeatable step) and the lattice site that the molecule then occupies is known as the *Halbkristall-Lage* (half-crystal position, growth position). The number of neighbors of a par-

ticle in the *Halbkristall-Lage* is exactly *half* of that of a particle in the interior of the crystal.

As far as the *wiederholbarer Schritt* is concerned, there is no difference between the various lattice planes of a crystal [4,6]. However, as one plane is completed a new one has to be started. A simplifying provision of the Kossel-Stranski theory is that all neighbors at a certain distance are equal. In reality, their bond energies are more or less utilized owing to their position. If this is disregarded it is easily demonstrated that a new layer of molecules will start from its middle and not from its edge, since the seed molecule will have more neighbors in the former case.

If a lone particle, settling on a face to begin an entirely new layer, has a great number of closest neighbors, it is obvious that this face will grow fast normally to itself, since new layers are easily nucleated.

If the corresponding neighbor numbers are recorded for the various lattice planes of a three-dimensional model, it is easy to arrange the faces in order of increasing normal growth velocity, *i. e.* in order of decreasing importance on the final crystal. The counting of the neighbors should be performed in a space lattice model of considerable size (20—30 unit cells).

In reality, the kinetics of crystal growth are more complicated than is provided in the Kossel-Stranski theory. Growth layers of visible thickness can be observed microscopically. They are easily studied in the electrodeposition of metals. They are about one micron thick and usually spread from a point in the interior of the crystal surface, though at high current densities from the edges. These thick growth layers are always to be found in the most close-packed surfaces. They are sometimes due to screw dislocations, a phenomenon that has played a great role in the literature on crystal growth lately [15].

CUBIC STRUCTURES

For cubic lattices the interplanar spacings d are calculated from the formula

$$d = a / \sqrt{h^2 + k^2 + l^2}$$

where a is the edge length of the unit cube and h, k, l the Miller indices of the surface. All sets of values h, k, l that are a multiple of 2, 3 or 4 of a former set should be excluded since they will correspond to higher order reflexions but not to physical lattice planes. For interlacing lattices certain other restrictions apply in addition. These are given in the table headings.

The reticular densities (number of atoms in the area a^2) are obtained from the relation $D = \frac{n}{V} \cdot d$ and are recorded in the second column in the tables. In the third column the number of nearest neighbors of a molecule starting a new plane is given. The last column, finally, gives the sequence according to column 3. In determining this order the following should be observed. Due to the rapid falling off of homopolar forces with distance, the expression $n_1/n_2/n_3$ may be considered as a kind of (decimal) fraction where one molecule in the first place corresponds to several in the second and so on. If, for instance, the

Table 1. Simple cubic lattice.

Restriction: h, k, l without common factor. $r_1 = a$; $r_2 = a\sqrt{2}$; $r_3 = a\sqrt{3}$.

Face *hkl*	Reticular density	Number of nearest neighbors	Sequence according to column 3
100	1	1/4/4	1 X
110	$1/\sqrt{2}$	2/5/2	2 X
111	$1/\sqrt{3}$	3/3/4	4 X
210	$1/\sqrt{5}$	2/6/4	3
211	$1/\sqrt{6}$	3/5/3	5 X
221	$1/\sqrt{9}$	3/5/4	6
310	$1/\sqrt{10}$	2/6/4	3

An X in the last column denotes the faces enumerated by Stranski and co-workers [7].

Table 2. Face-centered cubic lattice (cubic close-packing).

Restriction: All h, k, l even or all odd.

$r_1 = a/\sqrt{2} = 0.707\ a$; $r_2 = a$; $r_3 = a\sqrt{3}/\sqrt{2} = 1.225\ a$.

Face *hkl*	Reticular density	Number of nearest neighbors	Sequence according to column 3
111	$4/\sqrt{3}$	3/3/9	1 X
200 = 100	$4/\sqrt{4}$	4/1/12	2 X
220 = 110	$4/\sqrt{8}$	5/2/10	3 X
311	$4/\sqrt{11}$	5/3/10	4 X
331	$4/\sqrt{19}$	5/3/12	5
420 = 210	$4/\sqrt{20}$	6/2/10	6 X
422 = 211	$4/\sqrt{24}$	5/3/12	5
511	$4/\sqrt{27}$	5/3/12	5
531	$4/\sqrt{35}$	6/3/10	7 X
442 = 221	$4/\sqrt{36}$	5/3/12	5
620 = 310	$4/\sqrt{40}$	6/2/10	6

Table 3. Face-centered cubic elements.

Element	Mode of crystallization	Order of observed crystal faces compared to theories
Cu	Sublimation [19]	1 2 3 111, 100, 110 1 2 3
»	Solidification [19,23]	1 2 3 111, 100, 110 1 2 3
»	Electrodeposition	
	a) from simple salt solutions [19,20]	1 2 3 7 6 4 8 111, 100, 110, 211, 210, 311, 511 1 2 3 5 6 4 5
	b) from complex halides [21]	– – 1 3 *hk*0, *hkk*, 111, 110 – – 1 3 2
»	Cementation with Fe [19]	100 2
»	Natural crystals [19]	2 3 1 6 11 7 4 8 9 100, 110, 111, 210, 310, 211, 311, 511, 531 2 3 1 6 6 5 4 5 7
Ag	Sublimation [19]	1 2 10 111, 100, 221 1 2 5
»	Solidification [19]	1 2 3 111, 100, 110 1 2 3
»	Electrodeposition	
	a) from simple salt solutions [19,12,22]	1 2 7 3 5 – – 111, 100, 211, 110, 331, 751, 861 1 2 5 3 5 – –
	b) from ammoniacal solutions [17]	1 2 3 – 111, 100, 110, 720 1 2 3 –
	c) from complex halides and cyanides [17]	1 – 7 2 3 111, *hk*0, 211, 100, 110 1 – 5 2 3 4 2 1 –
	d) from molten salts [22]	311, 100, 111, 522 4 2 1 – 2 1
»	Reduction with SO_2 [19]	100, 111 2 1
»	Natural crystals [19]	2 1 3 6 11 – – – – 4 100, 111, 110, 210, 310, 410, 520, 740, 311 2 1 3 6 6 – – – – 4 1 2
Au	Sublimation [19]	111, 100 1 2
»	Solidification [19]	1 2 111, 100 1 2
»	Reduction with	
	a) $H_2C_2O_4$ [19], amyl alcohol [19], Na_2SO_3 [24]	1 111 1 2 1 3 – –
	b) HCHO [19], $FeCl_2$ [19]	100, 111, 110, *hkk*, *hkl* 2 1 3 – – 2 3 6 4 –
»	Amalgamation [19]	100, 110, 210, 311, 321 2 3 6 4 – 1 2 3 6 – 11 – 4 7
»	Natural crystals [19]	111, 100, 110, 210, 520, 310, 410, 311, 211 1 2 3 6 – 6 – 4 5

Pb	Sublimation [19]	1 111, 1	2 100 2					
»	Solidification [19]	1 111, 1	2 100 2					
»	Electrodeposition from simple salt solutions [19,22]	1 111, 1	2 100, 2	3 110, 3	4 311 4			
Pt	Gas phase reduction [19]	1 111, 1	2 100, 2	3 110 3				
»	Solidification [19]	1 111, 1	2 100 2					
»	Natural crystals [19]	2 100, 2	1 111, 1	3 110, 3	– 320, –	– 530, –	6 210, 6	11 310 6
γ-Fe	Solidification of pig iron [19]	1 111, 1	3 110, 3	2 100 2				
Ni	Electrodeposition from simple salts [25]	2 100, 2	1 111 1					

bonding energy is proportional to r^{-6}, as is often assumed [16], and if $r_2 = r_1\sqrt{2}$, one molecule in the first place will correspond to 8 in the second. If r_2 is only 10 % longer than r_1, the proportion will still be 1:1.8. Hence, we may safely adopt the following principle: The relative value of the expression $n_1/n_2/n_3$ is generally determined by n_1. If two expressions have the same n_1, their relative positions will be determined by n_2. If both the n_1:s and n_2:s are equal, n_3 will decide and so on.

The discrepancies between the two theories will be obvious from the last column of the tables. An X in the last column denotes the faces enumerated by Stranski and co-workers [7,8]. They are usually, but not always, given by them in the same order as here. It should also be noted that there are several misprints in the tables in the paper by Stranski and Kaischew [7] that are cited uncorrected by later writers [10,12,17,18]. One curious feature in the paper mentioned is that the authors [7] arbitrarily leave out some important low-indexed surfaces, particularly those that give the same expression in column 3 of the tables. This omission does not seem justified since these surfaces, *e. g.* 331, 211, 310 and 511 in the face-centered cubic lattice, are often observed on crystals (Table 3).

According to Stranski, any molecule on the surface of the crystal must at least be as strongly bonded as in the *Halbkristall-Lage*. His method to derive the equilibrium form is therefore to start from an arbitrarily simple form and substitute surfaces for corners and edges until the above provision is fulfilled. It appears that this method is arbitrary and is apt to exclude certain faces. Furthermore, the imagined removal of molecules from the corners and edges of the simple form has only to be carried so far that surfaces of the extension of a few tens of Ångström units are created. This does not necessarily imply that these surfaces will be detected by microscopic means on the final crystal. For instance, Stranski in a number of papers takes considerable pains to explain [16] why on body-centered cubic crystals the surface 211 appears but not the surface 111. It is now obvious from Table 4 that the former is theoretically

much more important than the latter and it will obviously be sufficient if 111 is present in submicroscopic extension.

In the first three lattices dealt with here, the spacing d assumes only one value for any sequence of parallel planes. This is not the case with the diamond cubic lattice (Fig. 1). An inspection of the space model will show that the 111 planes are spaced in a narrow-wide-narrow-wide sequence (Fig. 2). The identity spacing d obtained by X-ray analysis is $d = p + q$. This means that in determining the reticular density two narrow-spaced planes are considered to be one plane of double density. In applying the Kossel-Stranski theory, the molecule starting a new layer should be located at the distance q from the outer surface of the crystal (*e. g.* if the crystal surface is plane 2, the lone molecule will be in plane 3). The same condition is found with some other planes in

Table 4. Body-centered cubic lattice.

Restriction: $h + k + l = 2\ n$. $r_1 = a\sqrt{3}/2 = 0.866\ a$; $r_2 = a$; $r_3 = a\sqrt{2}$

Face *hkl*	Reticular density	Number of nearest neighbors	Sequence according to column 3
110	$2/\sqrt{2}$	2/2/5	1 X
200 = 100	$2/\sqrt{4}$	4/1/4	4 X
211	$2/\sqrt{6}$	3/3/5	2 X
310	$2/\sqrt{10}$	4/2/6	5
222 = 111	$2/\sqrt{12}$	4/3/3	6 X
321	$2/\sqrt{14}$	3/3/6	3
411	$2/\sqrt{18}$	4/3/6	7

Table 5. Diamond cubic lattice.

Restriction: $h^2 + k^2 + l^2 = 8\ n$ or $8\ n + 3$

$r_1 = a\sqrt{3}/4 = 0.433\ a$; $r_2 = a\sqrt{2}/2 = 0.707\ a$; $r_3 = a\sqrt{11}/4 = 0.829\ a$; $r_4 = a$

Face *hkl*	Reticular density	Number of nearest neighbors	Sequence according to column 3
111 D	$8/\sqrt{3}$	1/3/6/3	1 X
220 = 110	$8/\sqrt{8}$	1/5/5/2	2 X
311 D	$8/\sqrt{11}$	2/5/4/3	5 X
400 = 100	$8/\sqrt{16}$	2/4/6/1	4 X
331 D	$8/\sqrt{19}$	1/5/7/3	3
422 = 211	$8/\sqrt{24}$	2/5/4/3	5
511 D	$8/\sqrt{27}$	2/5/4/3	5
531 D	$8/\sqrt{35}$	2/6/5/3	7
620 = 310	$8/\sqrt{40}$	2/6/5/2	6 X

A "D" in the first column denotes a "double", non-coplanar face.

Table 6. Body-centered cubic and diamond cubic elements.

Element	Mode of crystallization	Order of observed crystal faces compared to theories
Body-c.cubic		
W, Mo, Ta	Sublimation [16]	$\overset{1}{1}10,\ \overset{2}{4}00,\ \overset{3}{2}11$
V	Reduction of V_2O_5 with Na [19]	$\overset{1}{1}10,\ \overset{2}{2}00$
α-Fe	Reduction of $FeCl_2$ with H_2 [19]	$\overset{1}{2}00,\ \overset{4}{1}10,\ \overset{-}{1}k0$
»	Electrodeposition from fused chlorides [26]	$\overset{2}{4}00$
Diamond cubic		
C (diamond)	Natural crystals [19]	$\overset{1}{1}11,\ \overset{2}{1}10,\ \overset{4}{1}00,\ \overset{-}{2}10,\ \overset{9}{3}10,\ \overset{6}{2}11,\ \overset{7}{5}11,\ \overset{12}{3}21$
Si	Reduction of $SiCl_4$ with Al [19]	$\overset{1}{1}11,\ \overset{2}{1}10$

the lattices dealt with below. Such "double" planes are marked "D" in the first column in the tables.

In Tables 3 and 6, the two theories are tested on elements with cubic structures. Most of the experimental data have been taken from P. Groth, Chemische Krystallographie [19], which contains an abundance of material that does not seem to have been utilized in this connection earlier. The third column in the tables gives the observed crystal faces in order of decreasing importance. Above and below the index figures, the ordinal numbers of the various faces according to Bravais' and Kossel-Stranski's theories are given with small figures. These numbers show the correlation between the two theories and experimental results.

HEXAGONAL STRUCTURES

In hexagonal lattices, the spacings d are obtained from the formula

$$\frac{1}{d^2} = \frac{4}{3a^2}(h^2 + k^2 + hk) + \frac{l^2}{c^2}$$

The hexagonal lattice is indexed with only three indices here. The fourth index i often given is related to h and k by the equation $h + k + i = 0$. In the hexagonal and following lattices, interplanar spacings are used instead of reticular densities. They are expressed in relative measure or in Ångström units.

In the hexagonal close-packed lattice, c equals $a\sqrt{8/3} = 1.633\ a$. Mg ($c = 1.627\ a$), Be and Ti ($c = 1.590\ a$) are close to the ideal value. In Cd ($c = 1.890\ a$) and Zn ($c = 1.855\ a$) the c-axis is relatively longer and the sequence of planes will be somewhat different. Earlier workers [11,28] treated Zn and Cd as ideally close-packed. It will be obvious from the tables, however, that the correlation with observation will be better if account is taken of the stretching of the c-axis.

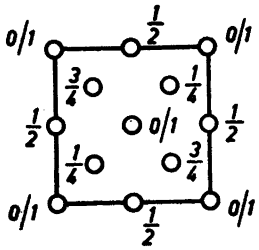

Fig. 1. Diamond cubic lattice, unit cell, basal projection. The figures denote the position of atoms above the basal plane.

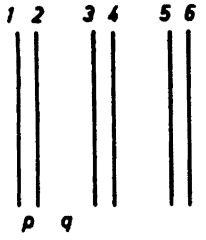

Fig. 2. Arrangement of the 111-planes of the diamond cubic lattice. $q = 3p$. $d = p + q$.

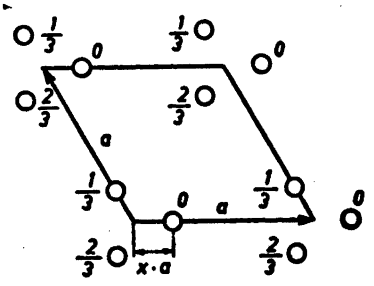

Fig. 3. Unit cell of Se and Te, basal projection. Position of atoms: $x00$, $0x\frac{1}{3}$, $\bar{x}x\frac{2}{3}$.

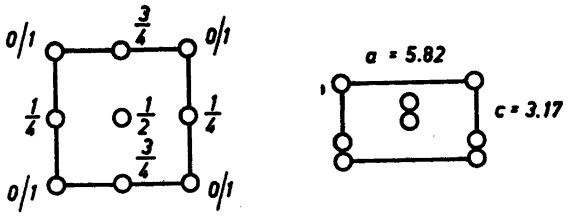

Fig. 4. Unit cell of white tin. a) Unit cell seen from top. b) Unit cell seen from side.

The elements selenium and tellurium have a more complex hexagonal structure. A basal projection of their unit cell is shown in Fig. 3. The lattice is built up of chains of atoms which are arranged in a spiral around an immaterial axis. The chains are perpendicular to the plane of the figure. Some data of importance are collected in Table 10.

These elements are of particular interest since deviations from the Kossel-Stranski theory have been reported with them. Stranski and co-workers [8] predicted the surfaces 101, 001, 102 and 100. Straumanis [11,29,30] in experiments on sublimated crystals of Se and Te did not find 001 and 102. The absence of the basis, 001, was particularly conspicuous. However, it seems clear that 001 should indeed *not*, according to the Kossel-Stranski theory, belong to the most important surfaces of the crystals. Since the closest neighbor is

Table 7. Hexagonal close-packed lattice (Be, Ti, Mg).

Restriction: $l = 2n$, if $h-k = 0$ or $3p$.

$r_1 = a$; $r_2 = a\sqrt{2}$; $r_3 = a\sqrt{8/3} = 1.633\ a$.

Face *hkl*	Interplanar spacing *d*	Number of nearest neighbors	Sequence according to column 3
100 D	0.866 *a*	4/4/0	3 X
002 = 001	0.816 *a*	3/3/1	1 X
101 D	0.765 *a*	4/2/1	2 X
102 D	0.594 *a*	5/2/1	5 X
110	0.500 *a*	5/2/0	4 X
103 D	0.461 *a*	5/3/1	6
112	0.427 *a*	5/3/1	6

Table 8. Stretched hexagonal close-packed lattice (Zn, Cd).

$c = 1.87\ a$; $r_1 = a$; $r_2 = 1.10\ a$; $r_3 = 1.49\ a$.

Face *hkl*	Interplanar spacing *d*	Number of nearest neighbors	Sequence according to column 3
002 = 001	0.935 *a*	0/3/3	1
100 D	0.865 *a*	2/2/4	3
101 D	0.786 *a*	2/2/2	2
102 D	0.636 *a*	2/3/2	4
103 D	0.506 *a*	2/3/3	5
110	0.500 *a*	3/2/2	6
112	0.441 *a*	3/2/3	7

Same result with six kinds of neighbors.

in the same chain the continuation of a chain is obviously more favored than the starting of a new chain. Since 001 consists of ends of chains it should grow fast and hence not appear in the final crystal. Actually, Straumanis [29] found the *c*-axis to be the preferred direction of growth and that needles along this axis often resulted. In the case of Se and Te, the *c*-axis is obviously the most important periodic bond chain vector in the sense of Hartman and Perdok [14]. On the other hand, the surfaces parallel to the chains, *i. e.* the prism faces, requiring nucleation of new chains for their normal growth, should grow slowly and thus be important surfaces in the final crystal. In fact, four out of five faces observed by Straumanis [29] on selenium were prism faces. Although the Kossel-Stranski theory cannot distinguish between the higher prism faces, Table 11 shows that it is in good agreement with the observed faces on selenium. The Bravais theory is somewhat less successful since it does not take full account of the favored length growth of the chains and thus gives the pyramid faces, 102, 111 *etc.* a too important position.

Table 9. Hexagonal close-packed elements.

Element	Mode of crystallization	Order of observed crystal faces compared to theories
Be	Reduction of $BeCl_2$ with Na [19]	2 1 5 3 4 001, 100, 110, 101, 102 1 3 4 2 5
Ti	Electrodeposition from fused salts [27]	2 1 001, 100 1 3
Mg	Sublimation [11,19]	2 3 1 001, 101, 100 1 2 3
Zn	Sublimation [11,28]	1 3 6 2 4 001, 101, 110, 100, 102 1 2 6 3 4
Cd	Sublimation [11,28]	1 2 3 6 – 001, 100, 101, 110, 111 1 3 2 6 –
Cd	Electrodeposition from simple salts [12,28]	1 2 3 001, 100, 101 1 3 2

Table 10. Lattice constants of selenium and tellurium.

	a	c	x	r_1	r_2	r_3	r_4
Se	4.36	4.95	0.217	2.32	3.46	3.67	4.36
Te	4.45	5.91	0.269	2.86	3.46	4.45	4.45

x is expressed as a fraction of a. *Cf.* Fig. 3. r_1 is the distance to the nearest atom in the same chain, r_2 to the nearest atoms in adjacent chains, r_3 is the distance to the nearest atoms in the same plane 001 and r_4 to the next nearest atom in the same chain. All distances in this and the following tables are expressed in Ångström units.

Table 11. Selenium lattice.

Restriction: 00l only with $l = 3n$.

Face hkl	Interplanar spacing d	Number of nearest neighbors	Sequence according to column 3
100	3.77	0/3/0/2	1 X
101 D	3.01	1/0/1/2	3 X
110	2.17	0/4/0/3	2
102 D	2.07	1/1/1/2	5 X
111 D	2.00	1/0/1/3	4
201 D	1.77	1/1/1/2	5
112 D	1.64	1/0/1/3	4
003 = 001		1/2/1/0	6 X
120	1.43	0/4/0/3	2
230		0/4/0/3	2

Observed surfaces on sublimated crystals [11,29]:

1 2 3 8 –
100, 101, 110, 120, 230
1 3 2 2 2

Table 12. Tellurium.

hkl	100	101	102	110	111	201
d	3.86	3.24	2.34	2.22	2.08	1.83

Mode of crystallization	Order of observed crystal faces compared to theories
Sublimation [11,20]	$\overset{1}{100},\ \underset{2}{\overset{1}{101}},\ \overset{2}{110},\ \overset{12}{114}$
Solidification [19]	$\underset{3}{101}$
Decomposition of K_2Te [19]	$\overset{1}{100},\ \underset{1}{\overset{2}{110}},\ \underset{2}{\overset{4}{111}},\ \overset{6}{201}$
Natural crystals [19]	$\overset{1}{100},\ \underset{1}{\overset{3}{101}},\ \underset{2}{\overset{6}{001}}$

The data derived from Groth [19] were transformed from rhombohedral to hexagonal lattice.

Table 13. Tetragonal tin lattice.

Restriction: $h + k + l = 2n$; $2\ k + l = 2n + 1$ or $4\ n$

$r_1 = 3.02$; $r_2 = 3.17$; $r_3 = 3.75$; $r_4 = 4.41$

Face *hkl*	Interplanar spacing *d*	Number of nearest neighbors	Sequence according to column 3
200 = 100	2.91	1/0/1/4	1
101 D	2.79	1/1/2/2	2
220 = 110	2.05	2/0/2/2	5
211 D	2.01	2/1/1/2	7
301 D	1.65	1/1/2/4	4
112	1.48	1/1/2/3	3
420 = 210	1.30	2/0/2/4	6
332	1.03	2/1/1/4	

Same result with five kinds of neighbors.

Observed sequence: Solidification [16]	$\overset{1}{100},$	$\underset{2}{\overset{2}{101}},$	$\underset{1}{\overset{3}{110}},$	$\underset{3}{\overset{6}{112}}$			
Electrodeposition from simple salts [19,22]	$\underset{2}{101},$	$\underset{1}{100},$	$\underset{3}{112},$	$\underset{-}{332},$	$\underset{5}{110},$	$\underset{4}{301}$	

In applying the Kossel-Stranski theory to the latter faces, one finds that they are spaced in a narrow-narrow-wide sequence. The lone atom considered should be across a wide spacing from the surface of the crystal since this will correspond to the bottle-neck in the growth process. Furthermore, different results may be obtained owing to the position of the surface in relation to the axis cross. The smallest value has been chosen, since this will correspond to the appearance of the surface on the final crystal. Often only every second of the pyramid faces are present on the crystals.

It has been assumed that the growth of Se and Te occurs by addition of atom by atom to the crystal. With some pyramid faces one would then find that as an atom is added to a chain it would have no nearest neighbor in the chain but just a hole below itself. Since this cannot be the case, one is led, on purely geometric grounds, to assume that the particles added are actually diatomic molecules. This is in accordance with the fact that selenium vapor consists of diatomic molecules.

Tellurium will obtain the same theoretical equilibrium form as selenium according to the Kossel-Stranski theory. Owing to other lattice dimensions the faces will appear in a somewhat different order according to Bravais as shown in Table 12. It is obvious that the agreement between theory and fact is good also for tellurium.

TETRAGONAL TIN STRUCTURE

White tin crystallizes in a tetragonal interlacing body-centered lattice, the unit cell of which is shown in Fig. 4 in basal and side projections. The spacings d are obtained from the formula

$$\frac{1}{d^2} = \frac{h^2 + k^2}{a^2} + \frac{l^2}{c^2}$$

The tin lattice may be considered as a diamond cubic lattice, compressed in the c direction. It is obvious from Fig. 4 b that the plane 101 is of the same nature as the octahedral plane 111 in the diamond cubic lattice, having alternating narrow and wide spacings.

The experimental data derived from Groth [19] (originally published by Miller in 1843) had to be recalculated by turning the axis cross 45° around the c-axis. While the two theories do not agree too well in the case of tin, experimental data correlate well with the nearest neighbors' theory.

ORTHORHOMBIC STRUCTURES

Sulfur and iodine crystallize in the orthorhombic system. Sulfur has a complicated face-centered unit cell with 128 atoms belonging to it. The space group restrictions are: All *hkl* even or all odd; $h + k = 4n$ in *hk*0 *etc.* Donnay and Harker [3] maintain that sulfur crystallizes in accordance with the Bravais theory. According to Hartman and Perdok [14] better results are obtained for sulfur with the reticular density theory, if the flat, stepped and kinked surfaces are considered separately. All the authors mentioned take account only of the appearance of the surfaces, not of their relative importance. The latter is given by Groth [19]. Correlation of his data with decreasing d-values, considering the above restrictions, shows the following:

Orthorhombic sulfur, natural crystals [34]:

1	3	4	2	14	7	6	5	11	9	10	8
111,	113,	011,	001,	010,	110,	115,	101,	112,	133,	013,	131

Orthorhombic sulfur, sublimated:

$$\overset{2}{001},\ \overset{10}{013},\ \overset{4}{011},\ \overset{14}{010},\ \overset{7}{110},\ \overset{1}{111},\ \overset{3}{113},\ \overset{6}{115},\ \overset{15}{117}$$

Orthorhombic sulfur, solidified from melt:

$$\overset{1}{111},\ \overset{4}{011},\ \overset{3}{113}$$

Orthorhombic sulfur, crystallized from solutions:

$$\overset{1}{111},\ \overset{14}{010},\ \overset{3}{113},\ \overset{2}{001},\ \overset{4}{011},\ \overset{7}{110},\ \overset{22}{100}$$

Iodine has a less complicated structure with 4 I_2-groups in the unit cell and with the following space-group restrictions: $h + k = 2n$ in hkl; $h = 2n$, $k = 2n$ in $hk0$; $h = 2n$, $l = 2n$ in $h0l$; $k = 2n$ in $0kl$. Iodine crystals, obtained in various ways, show the following surfaces in order of decreasing importance[19] and with the correlations with decreasing d-values noted: $\overset{2}{111}$, $\overset{1}{001}$, $\overset{3}{010}$, 313, 316, 110, 119. The last four faces will obtain ordinal numbers ranging between 10 and 20.

On the whole, these elements do not conform very well with the Bravais theory.

DISCUSSION OF RESULTS

It will be obvious from the tables that the two theories are in fair agreement for simple structures. They almost coincide for the face-centered cubic lattice and the stretched hexagonal close-packing (Zn, Cd). Kossel-Stranski's theory seems to correlate somewhat better with experimental results. However, it should be noted that this is fictitious to some extent, because this theory is less selective so that two or more planes will often obtain the same ordinal number. It is noteworthy that the agreement is best with metallic structures, although these, consisting of positive metal ions and a negative electron gas, are not strictly homopolar. With more complicated structures, such as those of sulfur and iodine, deviations from the Bravais theory are found, while the Kossel-Stranski treatment will be too intricate. In general, the Bravais theory is much easier to apply.

It is further obvious from the tables that the degree of correlation between theory and experiment is dependent on the conditions of the crystallization process. This is natural because both theories take account of only the lattice geometry and not the environment (diffusion, adsorption *etc*). Since both theories are energetic they imply conditions close to equilibrium, *i. e.* a small supersaturation, supercooling or polarization and hence generally a slow crystallization process. As the influence of the environment becomes more pronounced the crystals conform less well with the theories. It is natural that the best agreement is found with sublimated crystals: Sublimation is a slow process, occurring at high temperature, favoring equilibrium, and no foreign molecules are present to disturb the crystallization. On the other hand, in deposition from solutions, as by reduction agents (natural crystals seem to belong to this group) and sometimes by electrolysis, great disturbances and

deviations from the theories occur. It seems that the outer disturbances increase, *i. e.* the conformity with the theories decreases, somewhat in the following order: sublimation, solidification of melt, electrolysis of solutions of simple salts, chemical reduction, electrolysis of molten salts, electrolysis of complex salt solutions.

Electrodeposition as such, *i. e.* the superposition of an electric field on the lattice forces does not seem to influence the crystallization considerably, as is sometimes assumed [11,31]. If the electrocrystallization of metals is observed under the microscope it is often striking how little the direction of stream-lines means in comparison with the direction of preferred growth in the lattice. A crystal needle may grow not only at right angles to the general direction of the stream-lines but actually just opposite it, *i. e.* towards the *cathode*. The fact that electrocrystallization as such conforms so well with the theories of homopolar crystal growth supports the view that the particles that are built into the lattice on the cathode are neutral atoms, not ions [12].

The nature of the crystallizing particles seems to be of minor influence as long as the lattice is the same. For instance, Table 3 shows that, in general, Cu, Ag and Au develop the same surfaces, particularly on natural crystals [32].

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Determination of Small Amounts of Total Cholesterol by the Tschugaeff Reaction with a Note on the Determination of Lathosterol

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A simple sensitive method for the determination of cholesterol based on Tschugaeff's color reaction is described.

No color is obtained with dihydrocholesterol or epicholestanol. Coprosterol gave a faint reaction (absorbance about 1/10 of that of cholesterol).

Amounts of cholesterol as low as 0.004 mg per ml reaction mixture can be determined with an accuracy of about 3 %.

Δ-7-cholestenol (lathosterol) gives a yellow color reaction under the same conditions as cholesterol with absorbance maximum of 395 mμ and almost no absorbance at 528 mμ where cholesterol yields maximum absorbance.

The Tschugaeff reaction (with acetyl chloride and zinc chloride) [1] is the obvious means to use for the determination of small amounts of cholesterol in biological material, since it is much more sensitive than the Liebermann-Burchard reaction used by most investigators. The present method is a modification (and simplification) of the one originally described by Görtz [2,3] for use with blood.

EXPERIMENTAL

Reagents.

Aqueous KOH solution 33 %.
Petroleum ether, b. p. below 50° C.
Ethanol, absolute
Sodium sulfate, anhydrous, analytical grade.
Glacial acetic acid » » *
Acetyl chloride » » *
Chloroform » » *
Zinc chloride, anhydrous » » (sticks).

* These reagents must be miscible with each other without turbidity.

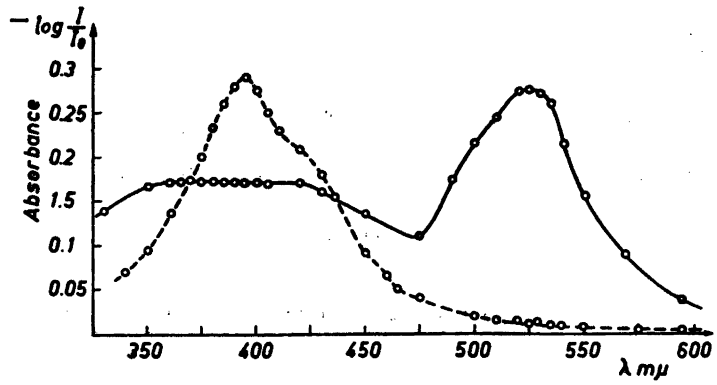

Fig. 1. Absorption curves. O———O *cholesterol, 0.0108 mg per ml reaction mixture.* O- - - -O *lathosterol, 0.0031 mg per ml reaction mixture.*

Zinc chloride solution in glacial acetic acid was prepared as follows.

40 g anhydrous $ZnCl_2$ (sticks, analytical grade) were quickly crushed and weighed in a dry 250 ml bottle and mixed with 153 ml glacial acetic acid. The bottle was shaken, stoppered and kept at 80° C for 2 ½ hours with occasional shaking. After cooling to room temperature the solution was filtered by suction through a glass filter.

The $ZnCl_2$-reagent was kept in a dark bottle with a tight glass stopper and could be used until it took up sufficient moisture to become turbid when shaken with chloroform. We were able to use the reagent at least 3 months.

Procedure. A. Extraction. The sample, *e. g.* 200—300 microliter bladder bile (or 2—3 ml fistula bile) or 0.5 g liver was placed in a 25 ml Erlenmeyer flask, then 33 % KOH and alcohol were added to make the mixture 17 % with respect to KOH, and 50 % with respect to C_2H_5OH. The mixture was heated on a steam bath either with the flask sealed with a glass ball, or, when the amount of cholesterol was particularly small, in a nitrogen atmosphere. In case of bile the time of heating was about ½ hour, tissue was heated 2 to 3 hours according to the fat content and time required for homogenization.

After cooling to room temperature each sample was extracted three times with 5 ml portions of petroleum ether (b. p. below 50° C). The extracts were combined and washed with water until neutrality to lithmus. The combined extract was now dried over anhydrous Na_2SO_4, and filtered by suction, the sodium sulfate being washed with petroleum ether. The anhydrous solution was evaporated *in vacuo* at 40—50° C, and the residue transferred with chloroform into a 10 ml test tube. The main part of the chloroform was then evaporated on the steam bath to give a final volume of about 2 ml.

B. Color reaction. To each of the above tubes, containing about 2 ml chloroform solution, was added 1 ml of the $ZnCl_2$-reagent and 1 ml of acetyl chloride. The addition of acetyl chloride was most conveniently done with a burette. The contents of the tubes were then mixed with a glass rod, and the tubes were placed in a water bath at precisely 65° C for exactly 15 minutes. Thereafter the tubes were cooled in ice water and the reaction mixture was transferred to a 5 ml volumetric flask; the tubes were washed several times with small amounts of chloroform to bring the volume in the flask up to the 5 ml mark. The eosin-red solution was then placed in a 10 mm cuvette and the light absorbance (density, $d = -\log \frac{I}{I_0}$) was measured within 30 minutes at 528 mμ in a Beckman spectrophotometer. The same reaction was carried out with 2 ml of a standard solution of cholesterol in chloroform (*e. g.* 0.02 mg/ml) and with 2 ml chloroform as a blank.

Because of the sensitivity of the reaction to variations in the permissible low concentration of water, it is advisable to use a standard with cholesterol for each series of determinations.

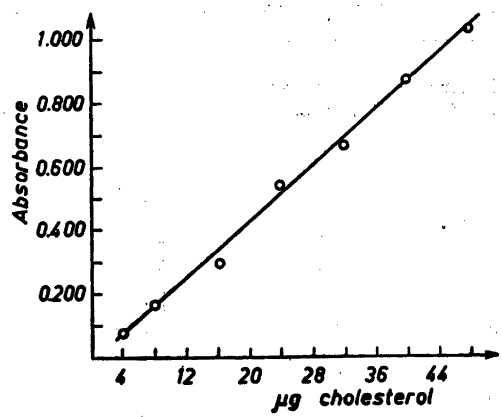

Fig. 2. Absorbance of cholesterol at 528 mµ after reaction with $ZnCl_2$-AcCl.

DISCUSSION OF THE METHOD

Development of the color at 65° C in 15 minutes was found more satisfactory than at 70° C in 12 minutes (as by Görtz [3]) or at 50° C in 20 minutes.

The maximal light absorbance was at 528 mµ. Fig. 1 shows the light absorbance curve found for 0.0108 mg cholesterol per ml reaction mixture and the corresponding curve for 0.0031 mg lathosterol. The developed color follows Beer's law in the concentration range from 0.004 to 0.048 mg cholesterol per ml reaction mixture as shown in Fig. 2.

We found that the proportion of the reagents mentioned gave the greatest color stability. The stability of the color is shown in Fig. 3 *.

Table 1 shows the results obtained by the present method and by the method of Sperry and Webb [4] as applied to hamster liver. The extraction of samples was carried out as described. The same extracts were used for both

Table 1. Comparison of the Sperry-Webb method with the $ZnCl_2$-AcCl-method.

mg cholesterol/ml extract	
Method of Sperry and Webb	Our method
0.0172	0.0188
0.0387	0.0382
0.0767	0.0758
0.0956	0.0963

* The authors thank Dr. S. Hartmann for making this curve by means of an automatically recording spectrophotometer.

Table 2. A series of routine cholesterol determinations with the $ZnCl_2$-AcCl-method.

Sample No.	Cholesterol mg per 5 ml	Light absorbance	Light absorbance per mg	Deviation from mean of single determination %
1	0.060	0.263	4.383	− 2.72
2	0.060	0.265	4.417	− 1.93
3	0.060	0.258	4.750	+ 5.22
4	0.024	0.107	4.458	− 0.99
5	0.024	0.110	4.583	+ 1.76
6	0.024	0.103	4.292	− 4.89
7	0.022	0.097	4.409	− 1.98
8	0.022	0.098	4.455	− 1.05
9	0.022	0.105	4.773	+ 5.68
10	0.018	0.078	4.333	− 3.89
11	0.018	0.083	4.611	+ 2.37
12	0.018	0.082	4.556	+ 1.18
		average	4.502	

methods, but with our procedure we used aliquots only one-tenths as large as those used for the Sperry-Webb determinations.

At the same occasion it was shown that the specific light absorbance at our method was 8 times that of the Liebermann-Burchard reaction used in the Sperry-Webb method.

ACCURACY OF THE METHOD

From a standard solution of cholesterol in chloroform different known aliquots were taken and the described procedure was carried out. The results are shown in Table 2.

The mean square error of the single measurement is $\sqrt{\frac{\Delta^2}{n-1}} = \pm 0.155$.

The mean square error of the average of the measurements is $\sqrt{\frac{\Delta^2}{n(n-1)}} = \pm 0.044$.

SPECIFICITY OF THE REACTION

In Table 3 are given the absorbances obtained with cholesterol, some cholesterol esters and some other sterols.

Dihydrocholesterol and epi-cholestanol gave no color.

Mixtures of cholesterol and dihydrocholesterol in varying proportions gave absorbances proportional to the amount of cholesterol.

Two samples of coprosterol prepared from different materials gave a faint reaction with absorption maximum at the same wave length as cholesterol but with absorbance of about 1/10 of that of cholesterol.

Mixtures of cholesterol and coprosterol in varying proportions gave absorbances lying on a straight line between the absorbances of the two substances.

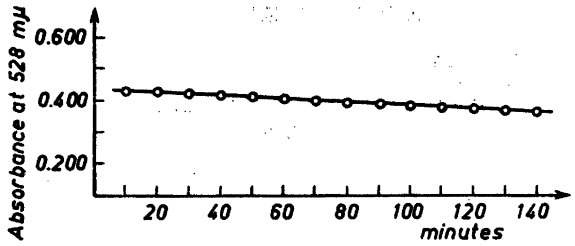

Fig. 3. The effect of time on the color stability of the reaction at 20° C. (Determined by means of a recording spectrophotometer).

The following bile acids: cholic, desoxycholic, chenodesoxycholic, glycocholic and taurocholic acid gave a yellowish-brown color reaction; the maximal absorbance of these acids was in the interval 375—450 mμ.

Δ-7-cholestenol, "lathosterol", gave a yellow color with a maximal light absorbance at 395 mμ (Fig. 1) and almost no absorbance at 528 mμ. The absorbance at 395 mμ follows Beer's law at least in the concentration interval 0.003—0.028 mg lathosterol/ml of the final mixture. Mixtures of cholesterol and lathosterol gave absorbances lying on a straight line. The present method may be utilized for simultaneous determination of cholesterol and lathosterol.

Table 3. Absorbance obtained with cholesterol, cholesterol esters, and some other sterols.

Compound	Melting point °C found	Melting point °C in literature	mg/ml	Light absorbance at 528 mμ	Absorbance mmole/ml
Dihydrocholesterol *	141.5	140.5	0.0197	0.000	0
Epi-cholesterol *	182	183	0.0190	0.000	0
Coprostanol **	101—102	100—102	0.0197	0.054	1 066
Coprostanol ***	100—101	100.5	0.0197	0.047	930
Cholesterol ****	148.0	147	0.0197	0.462	9 593
Cholesterylacetate	115.3	114.5	0.0208	0.308	8 349
Cholesterylpalmitate	88.3	90	0.0179	0.254	8 868
Cholesterylbenzoate	145—146	145.5	0.0227	0.455	9 837
"Phytosterol" (Merck)	94—97	—	0.0237	0.315	—
Stigmasterol (Merck)	168.8	170	0.0100	0.338	13 949
Stigmasterol (La Roche)	167.9	170	0.0214	0.537	10 356
Δ-7-Cholestenol (lathosterol) *****	123—125	123—124.5	0.0288	(0.020)	(268)

* Gives no color reaction with $ZnCl_2$-AcCl in chloroform.

** Isolated from human feces, purified through the digitonide after treatment with bromine.

*** Isolated from dog feces.

**** Purified through the dibromide.

***** The authors thank Professor L. Fieser for furnishing the sample of lathosterol.

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A Method for the Estimation of the Taurine Content and its Conjugation with Cholic Acid in Rat Liver. Bile Acids and Steroids 22

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A method for the determination of the taurine content of rat liver and for the taurine conjugation with cholic acid in rat liver homogenates is described. The taurine content of fresh rat liver was found to be 0.1—0.6 mg/g in male rats, and 0.7—1.1 mg/g in female rats.

Under the conditions described 2.2 μmole taurocholic acid was formed per g fresh liver. Due to the relatively high taurine content of the rat liver, addition of taurine to the reaction mixture had only an insignificant effect on the yield of taurocholic acid.

Homogenates centrifuged free from cell debris and nuclei were twice as effective as the total homogenates.

The taurine conjugation and the α-hydroxylation of desoxycholic acid have been effected by rat liver slices [1] and homogenates [2].

The present paper is primarily concerned with a simplified procedure to measure the taurine concentration and taurine conjugation in rat liver homogenates with the aid of ^{35}S-labelled taurine **.

The measurements of taurocholic acid formed was based on a selective extraction of taurocholic acid with *n*-butanol while the free taurine remained almost quantitatively in the water phase. Some of the factors influencing the synthesis of taurocholic acid from taurine have also been investigated and are reported here.

EXPERIMENTAL

The ^{35}S-labelled taurine had an activity of approximately 400 000 c/min./mg as measured in infinite thin layer with a TG 2 Tracerlab tube.

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** The author wishes to express his appreciation to L. Eldjarn and P. Fritzson, *Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway*, for the synthesis of ^{35}S-labelled taurine [3].

Albino rats, weighing 200—250 g, were used. The animals were killed by a blow on the neck. The livers were immediately removed and dropped into ice-cold isotonic KCl solution and weighed by differences.

The homogenate was prepared in a glass homogenizer with a loose-fitting pestle according to the method of Bergström and Gloor [3]. The homogenizing medium consisted of sucrose, potassium phosphate buffer of pH 7.4, magnesium chloride and nicotinamide [4]. One part of liver and three parts of homogenizing medium were homogenized for 45 seconds. The resulting homogenate was either used as such, or centrifuged free from cell debris and nuclei at 400 × g for 5 minutes.

In the incubation experiments 2 ml of the homogenate were added to each vessel with the appropriate additions. The total volume per vessel was 2.5—2.8 ml. The following concentrations were maintained in the incubation experiments: Potassium phosphate buffer (pH 7.4) 0.034—0.037 *M*, nicotinamide 0.016—0.018 *M*, $MgCl_2$ 0.004 *M*, sucrose 0.068—0.076 *M*, KCl 0—0.022 *M*.

The vessels were shaken in a Warburg apparatus at 37—38° C with a frequency of 100 strokes per minute. The gas phase was air and the incubation time was two hours.

The reaction was stopped by immersing the vessels for half a minute in boiling water. Much of the proteins was precipitated by this treatment. After cooling to room temperature, 1/3 volume of *n*-butanol was added and the vessels were shaken for 15 minutes in a shaking machine to establish equilibrium between the phases. The contents of the vessels were then centrifuged in narrow tubes at 400 × g for two minutes in order to obtain a clear separation of the two phases with the precipitated proteins at the interphase.

A suitable aliquot of the upper butanol layer (0.05—0.1 ml) was finally pipetted off for paper chromatography or plating. In the latter case empirical corrections of the counting data for self-absorbtion were made.

Analytical methods. The analytical technique was essentially based on the advantageous distribution of the taurocholic acid between the water and butanol phases. As the butanol and the water are partially miscible, it was found convenient to determine empirically the correlation between the original concentration in the water phase before extraction, and the concentration in the butanol phase after extraction with 1/3 volume *n*-butanol.

The analytical technique was tested by means of the quantitative paper chromatographic method as described by Eriksson and Sjövall [5,6].* In this system the taurine migrates with a speed approximately 1/3 of that of taurocholic acid. These compounds could therefore easily be separated.

The butanol phase could be applied directly on the paper, since the dry matter was only 5—6 mg/ml butanol. A sample of 0.05—0.1 ml was placed on the paper in small portions under a stream of warm air. After chromatography the spots were eluted with absolute alcohol and the eluate evaporated to a small volume for plating, or to dryness for spectrophotometric measurement in concentrated sulphuric acid at 389 mμ, using a Beckman quartz spectrophotometer.

Table 1 shows the results of some of these control experiments. It appears that the addition of radioactive taurine corresponding to 30 000 c/min. to 2.8 ml gave rise to only 15—20 c/min./0.05 ml butanol phase. It is also evident that no detectable amount of any extractable radioactive compound other than taurocholic acid had been formed. The slightly higher activity of the chromatographed sample was due to the smaller self-absorbtion, as most of the impurities in the butanol disappeared during the chromatographic procedure.

Further there is a close proportionality between the taurocholic acid formed and the amount of labelled taurine conjugated, the former determined spectrophotometrically, the latter by plating and counting of the butanol phase.

The effect of the pH on the distribution of the taurocholic acid between the water- and the butanol phases was studied. For this purpose ^{35}S-labelled taurocholic acid was prepared biologically as described above. Aliquots were dissolved in phosphate buffers of varying pH and shaken with 1/3 volume of butanol. Table 2 shows the resulting distri-

* The author is indebted to S. Erihsson and J. Sjövall, Department of Physiological Chemistry, University of Lund, for the use of their unpublished method for the quantitative determination of bile acids by paper chromatography.

Table 1. Total volume 2.8 ml. Vessels A—C, 2 ml 25 % homogenate centrifuged at 400 × g for 5 minutes. Vessel D, 2 ml 25 % uncentrifuged homogenate.

Vessel	Additions	c/min. in 0.05 ml butanol phase, directly plated	c/min. in 0.05 ml butanol, chromatographed, eluted and plated	μmole of added taurine ^{35}S conjugated	μmole taurocholate formed determined spectrophotometrically
A	0.8 μmole taurine-^{35}S = 30 000 c/min.	18	1	0	0
B	0.8 μmole taurine-^{35}S 2 μmole cholic acid	381	421	0.31	1.1
C	0.8 μmole taurine-^{35}S 2 μmole cholic acid	401	—	0.32	1.1
D	0.8 μmole taurine-^{35}S 2 μmole cholic acid	213	—	0.17	0.55

Table 2. The distribution of taurocholic acid between water and butanol at varying pH. 1.75 ml H_2O (0.75 ml 0.11 M phosphate buffer + 1 ml 1.15 % KCl) and 0.58 ml butanol (1/3 volume).

pH	c/min. added to each vessel	c/min. recovered in 0.1 ml butanol	Partition coefficient butanol/water	Concentration in butanol phase/initial conc. in H_2O
1	3 000 = 171/0.1 ml	468	~ 12	2.75
6.5	3 000	336	~ 8	2.0
7.2	3 000	344	~ 8	2.0
8	3 000	353	~ 8	2.0
14	3 000	342	~ 8	2.0

Table 3. The recovery of increasing amounts of taurocholic acid by butanol extraction. 0.75 ml potassium phosphate pH 7.2, 1 ml 20 % centrifuged homogenate, radioactive taurocholic acid corresponding to 1 600 c/min./vessel. 0—3 μmole inactive taurocholic acid added.

μmole inactive taurocholate added	c/min. recovered per 0.1 ml butanol phase
0	186
0.1	169
0.3	197
0.5	197
1.0	171
2.0	175
3.0	162

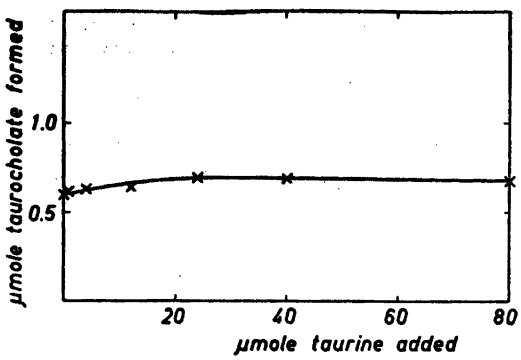

Fig. 1. Total volume 2.5 ml. 2 ml 25 % homogenate centrifuged at 400 g for 5 min. Substrate additions 2.5 µmole cholic acid, 0—80 µmole taurine. 0.05 ml butanol phase chromatographed, eluted and measured spectrophotometrically in concentrated H_2SO_4.

bution. By acidifying the solution, a greater concentration of taurocholic acid was achieved in the butanol phase. Nevertheless, we preferred to extract without acidifying the solution, as the addition of acid greatly affected the rate of migration of the bile acids on the paper chromatograms.

Table 3 shows the control of the distribution coefficient in the concentration interval used in the experiments to be described. Homogenate and a constant amount of ^{35}S-labelled taurocholic acid (0.2—0.3 µmole per vessel) were added to the vessels. Further there were added increasing amounts of inactive taurocholic acid. It appears that a constant concentration relationship between the butanol and water phases was obtained. Consequently there was no trapping of taurocholic acid in the precipitated proteins, and the partition coefficient also remained constant within the concentration range studied.

THE ESTIMATION OF FREE TAURINE IN RAT LIVER

As we could follow the formation of taurocholic acid in the rat liver homogenate both spectrophotometrically and by means of the ^{35}S-labelled taurine, we had the opportunity to calculate the taurine content of rat liver. Thus in the experiment shown in Table 1, only 0.32 µmole of the added 0.8 µmole labelled taurine had been conjugated, although the total amount taurocholic acid formed was 1.1 µmole, as determined spectrophotometrically (Table 1, B and C). To each vessel there were added 2 ml of a 25 % homogenate, *i. e.* 500 mg fresh liver. The amount of preformed taurine in the liver could consequently be calculated as follows:

$$\frac{(1.1-0.32) \times 0.8}{0.32} = 1.95 \text{ µmole/500 mg liver} = 0.49 \text{ mg taurine/g fresh liver.}$$

Similar calculations were performed in nine experiments with male rats. The taurine content was found to range between 0.1—0.6 mg/g liver (average 0.35 mg/g).

Three experiments with female rats gave a taurine content of 0.7—1.1 mg/g liver (average 0.9 mg/g). There were great variations in the values, but a sex difference seemed to be present.

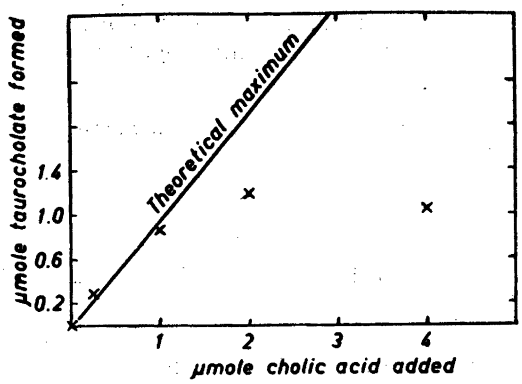

Fig. 2. Total volume 2.5 ml, 2 ml 25 % homogenate centrifuged at 400 g for 5 min. Substrate additions 1.6 µmole taurine, 0—4 µmole cholic acid. 0.05 ml butanol phase chromatographed, eluted and measured spectrophotometrically in 1 ml concentrated sulphuric acid.

This result seems to be in contrast with the findings of Sloan-Stanley [7] and of Blaschko *et al.*[8] who found the cysteic acid decarboxylase activity to be much higher in the male rat liver than in the female rat liver. This discrepancy may be another indication that taurine is formed through other metabolic channels than by the decarboxylation of cysteic acid [9-11].

The taurine content in male rat liver was reported by Wu [12] to be 0.104 ± 0.09 mg/g liver on a standard diet. After nine dayes fasting the content had increased to 0.6 ± 0.14 mg/g.

The relative high taurine content of the rat liver may also explain the insignificant effect of added taurine on the yield of taurocholic acid in the experiment shown in Fig. 1. This experiment was performed with inactive taurine and spectrophotometric determination of the taurocholic acid formed. Addition of 20 µmole taurine or more had only a weakly stimulating effect. This indicates that the preformed 2—3 µmole taurine present in the homogenate was enough to give optimal conditions for the reaction.

Fig. 2 illustrates the effect of increasing amounts of cholic acid on the synthesis of taurocholic acid. The amount formed levelled off at about 1 µmole cholic acid added. No taurine-conjugated or free cholic acid seemed to be present in the liver.

COMMENTS ON THE TAURINE CONJUGATION

The taurine conjugation and 7-hydroxylation of desoxycholic acid in liver slices and liver homogenates have been reported by Bergström *et al.*[1,2]

In preliminary experiments we were able to show that also cholic acid was conjugated in liver slices when incubated in Ringer buffer solution. In Table 1 is shown that rat liver homogenates effected conjugation of cholic acid with taurine, and that this conjugation was doubled when the homogenates were

centrifuged free from cell debris and nuclei at 400 × g for five minutes (Table 1, C and D). This result warrants further investigations on the localization of the process within the cell.

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Studies on the Conjugation of Cholic Acid with Taurine in Cell Subfractions. Bile Acids and Steroids 23

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The conjugation of cholic acid with taurine has been studied in subfractions of rat liver homogenate.

The full conjugating activity of the total homogenate was retained in the crude supernatant (III) after centrifugation at 12 000 × g for 10 minutes. The microsomic fraction (IV) and the particle-free supernatant (V) were each inactive, but recombination restored the full activity when ATP was added. Partial reactivation was achieved when fresh microsomes were incubated with boiled supernatant and ATP, or with an acetone powder of the particle-free supernatant (V), DPN and ATP.

The conjugation in crude supernatant (III) was stimulated by nicotinamide, DPN, ATP, Mg^{++}, the complex forming agent versene and possibly by TPN.

At low taurine and cholic acid concentrations, the conjugation was almost quantitative.

When ATP was omitted cyanide strongly inhibited the conjugation. When ATP was added, cyanide had no effect.

Recently we have published a simple method to follow the formation of taurocholic acid from cholic acid and ^{35}S-labelled taurine together with preliminary results on the conjugation in liver homogenates [1]. The present paper deals with some further studies on this process in cell subfractions.

EXPERIMENTAL

The experimental procedure previously reported [1] has been slightly modified for these studies.

Due to the higher taurine content in the female rat liver [1], we have used male albino rats weighing 200—250 g throughout the study. The homogenization of the liver was performed as previously described [1,2]. Four parts of homogenizing medium were used to one part of liver (crude homogenate = I). The homogenizing medium consisted of 5 parts of 0.11 *M* potassium phosphate buffer of pH 7.7 and 7 parts of 10 % sucrose.

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In the fractionation experiments, nuclei and cell debris were taken down by centrifugation at 400 × g for 5 minutes (homogenate = II), mitochondria at 12 000 × g for 10 minutes (crude supernatant = III), and the microsomic fraction at 100 000 × g for 30 minutes (IV), leaving in solution the particle-free supernatant (V). The mitochondria were washed once by resuspension in homogenizing medium and recentrifugation. When recombination of the different fractions was undertaken, the microsomic and the mitochondrial fractions were resuspended in homogenizing medium to 1/10 of the original homogenate volume and pipetted as such.

In all the experiments 1 ml of homogenate was used per vessel. Additions to the following final concentrations were made: nicotinamide 0.017 *M*, MgCl 0.00065 *M*, versene (ethylenediamine tetraacetate) 0.00075 *M*. The mixture was finally brought to a volume of 1.75 ml with a 1.15 % solution of potassium chloride. Incubation, protein precipitation and butanol extraction were performed as previously described [1].

In order to evaluate the relative amount of taurocholic acid formed, ^{35}S-labelled taurine * was added to the incubation mixture, and the radioactivity of taurocholic acid formed was determined. The absolute amount of taurocholic acid formed was evaluated from the amount of preformed taurine present in the homogenate batch used [1]. The results are given in micromoles taurocholic acid formed per vessel, *i. e.* per 200 mg of liver. Duplicates were always run.

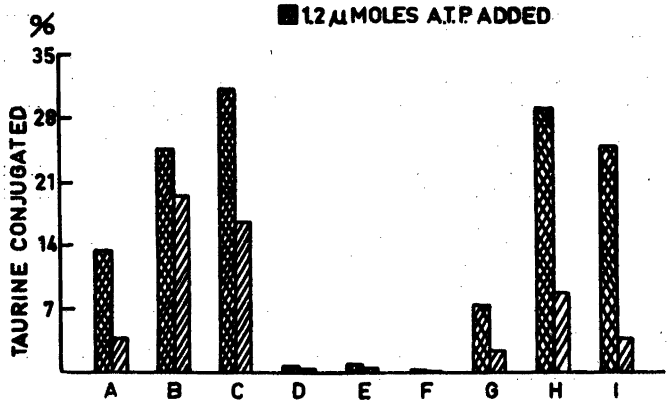

Fig. 1. Conjugation of cholic acid with taurine in liver cell subfractions.

Substrate additions: 2.5 μmole cholic acid, 2 μmole taurine. Further 1.2 μmole ATP where noted.

Vessels A. Total homogenate.

B. Homogenate centrifuged at 400 g for 5 min.
C. Homogenate centrifuged at 12 000 g for 10 min.
D. Homogenate centrifuged at 100 000 g for 30 min. (particle-free).
E. Sediment after centrifugation at 12 000 g (mitochondria).
F. Sediment after centrifugation at 100 000 g (microsomes).
G. Particle-free supernatant + mitochondria.
H. Particle-free supernatant + microsomes.
I. Particle-free supernatant + mitochondria + microsomes.

* The authors wish to express their appreciation to L. Eldjarn and P. Fritzson, *Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital*, Oslo, Norway, for the synthesis of ^{35}S-labelled taurine.

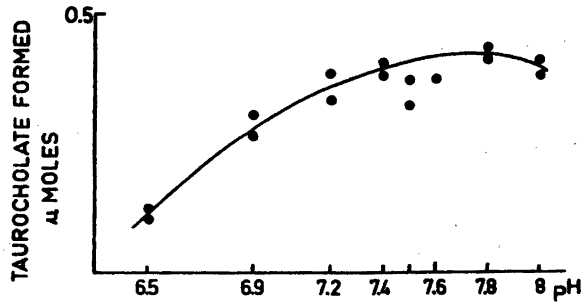

Fig. 2. Effect of pH. Crude supernatant + the following additions: 0.4 μmole ^{35}S-taurine, 1.0 μmole cholic acid, 1.2 μmole ATP.

RESULTS

The results from experiments with cell subfractions obtained by differential centrifugation are shown in Fig. 1.

The conjugating enzyme system was found to be localized in the crude supernatant fraction (III) of the liver cell, as the homogenate freed from nuclei and mitochondria by centrifugation at 12 000 × g for 10 minutes showed full activity (Fig. 1 C).

Neither the microsomic fraction alone (F) nor the particle-free supernatant (D) showed any activity. Full activity was obtained after recombination of these fractions in the presence of ATP (H).

We have performed several experiments with isolated microsomes without obtaining any conjugation of the added cholic acid and taurine. Addition of ATP, magnesium and coenzyme A was without any influence. Unfortunately the only preparation of coenzyme A available was a very crude extract. The possible role of coenzyme A will be subject to further studies.

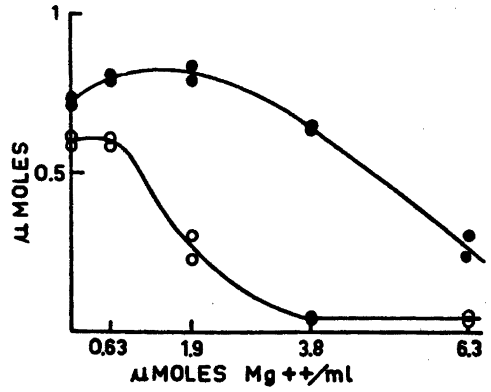

Fig. 3. Effect of Mg^{++}. Crude supernatant + the following additions: 0.4 μmole ^{35}S-taurine, 1.0 μmole cholic acid. Further with 1.2 μmole ATP (●), and without ATP (○). Magnesium concentrations 0—0.0065 M.

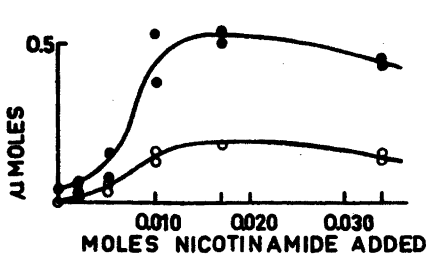

Fig. 4. Effect of nicotinamide. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid, 1.2 µmole ATP were indicated (●). No ATP (○), nicotinamide concentrations 0—0.034 M.

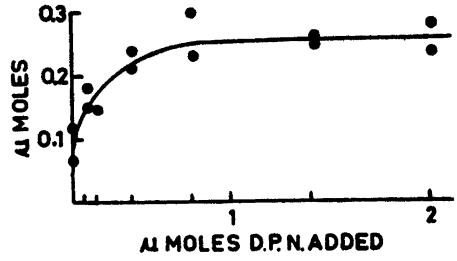

Fig. 5. Effect of DPN. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid. 0—2.25 µmole DPN per vessel.

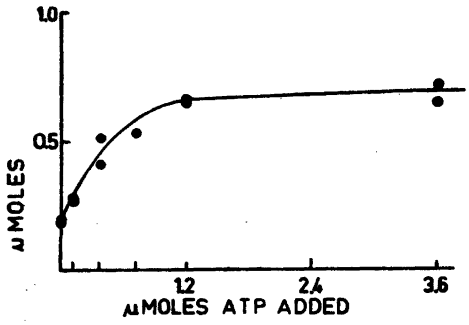

Fig. 6. Effect of ATP. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid. Nicotinamide conc. 0.017 M, *0—3.6 µmole ATP per vessel.*

Effect of pH. No distinct pH-optimum was found (Fig. 2). Homogenates were almost equally effective when homogenizing media of pH 7 to pH 8 were used. At lower and higher pH values the activity decreased. Therefore a potassium phosphate buffer of pH 7.7 was used throughout. In these experiments the pH changed from 7.7 to 7.3—7.2 during the incubation period.

Effect of Mg-ions. Magnesium had a weak but distinct stimulating effect on the conjugation in the presence of ATP. Without addition of ATP the effect was purely inhibiting (Fig. 3). We also tried the effect of manganese chloride, since Ernster [3] and Lindberg have recently shown manganese to have a stimulating effect on the regeneration of energy rich phosphate bonds. In our experiment the addition of 1 µmole $MnCl_2$ gave a 90 % inhibition.

Effect of nicotinamide, DPN, ATP, and TPN. Figs. 4, 5, 6 and Table 1 show the effects of nicotinamide, DPN and ATP on the conjugation when the three other factors were excluded. As ATP had no, or only a very small effect when nicotinamide was omitted, the effect of ATP was investigated in the presence of this compound.

All these experiments are made with a crude supernatant(III). Consequently no mitochondria were present. Since nicotinamide and DPN show such a marked effect, however, there must still be enzymes present in the microsomes

or the particle free supernatant capable of producing the necessary energy for the conjugation.

We have also tried the effect of TPN and found it to have a stimulating effect of approximately the same order as DPN. Our TPN preparation was very impure, containing only 6 % of the compound. Presumably DPN was one of the main contaminants.

Table 1 shows the effect of the different combinations of nicotinamide, DPN and ATP. The data indicate that ATP requires the presence of nicotinamide or DPN in order to show any significant effect. In the presence of nicotinamide and ATP, the addition of DPN gave no further stimulation. We did not find any significant effect when cytochrom c or fumarate were added alone or in combinations with the other factors tried.

Table 1. Effect of stimulating and inhibiting factors. Crude supernatant — 2 µmole taurine, 2 µmole cholic acid.

1.2 µmole ATP	1.5 µmole DPN	Nicotinamide 0.017 *M*	Cyanide (8.5×10^{-4} *M*)	Dinitrophenol (2.8×10^{-4} *M*)	1 µmole Taurocholate	µmole Taurocholate formed	
—	—	—	—	—	—	0.06	0.06
+	—	—	—	—	—	0.05	0.06
—	+	—	—	—	—	0.39	0.39
—	—	+	—	—	—	0.40	0.40
—	—	+	+	—	—	0.12	—
—	—	+	—	+	—	0.38	0.27
+	+	—	—	—	—	0.45	0.49
—	+	+	—	—	—	0.72	0.70
+	—	+	—	—	—	0.85	0.85
+	+	+	—	—	—	0.84	0.84
+	—	+	+	—	—	—	0.77
+	—	+	—	+	—	0.74	0.77
+	—	+	—	—	+	0.37	0.31

When compared with the results of Cohen and McGilvery [4] in their work on the *p*-aminohippuric acid synthesis in rat liver homogenates, several dissimilarities appear. These authors found a purely inhibiting effect with nicotinamide. DPN and ATP added together inhibited their system if fumarate was omitted, but both DPN and ATP stimulated when added alone.

Our results seem to be in good accordance with the findings of Mann and Quastel [5] who showed that nicotinamide protects DPN against a DPN-splitting enzyme in liver and brain, and with the findings of Rowen and Kornberg who showed that the following reactions take place in the liver

1. Nicotinamide + ribose-1-phosphate → nicotinamideriboside
2. Nicotinamideriboside + ATP → nicotinamidemononucleotide [6]
3. Nicotinamidemononucleotide + ATP → DPN + pyrophosphate [7]

The form of the curve illustrating the nicotinamide effect (Fig. 4) also indicates that in this case a more complex mecanism was involved than when DPN was added (Fig. 5).

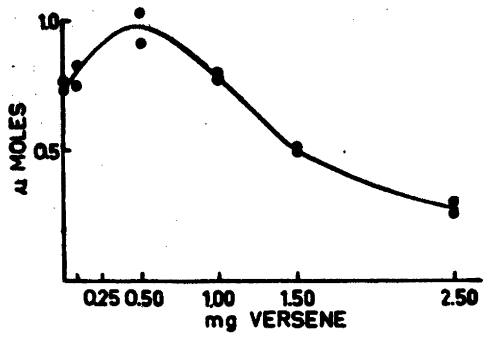

Fig. 7. Effect of versene. Crude supernatant + the following additions: 0.4 μmole ^{35}S-taurine, 1.0 μmole cholic acid, 1.2 μmole ATP, versene 0—2.5 mg per vessel.

Table 1 also shows that cyanide had a strongly inhibiting effect when ATP was omitted, whereas 2,4-dinitrophenol was almost without any effect in the concentration used (2.8×10^{-4} *M*). The addition of sodium taurocholate (1 μmole) gave a 50 % inhibition. The icterus-promoting compound icterogenin [8] was tried, but was found to have no effect.

Effect of versene. Fig. 7 shows that the addition of the complexforming agent versene had a positive effect on the formation of taurocholic acid, presumably by binding heavy metal impurities in the medium. Addition of more than 0.5 mg (7.5×10^{-4} *M*), gave an inhibiting effect. A similar effect of versene on the 7 α-hydroxylation of taurodesoxycholic acid has earlier been observed [9].

Effect of taurine and cholic acid. In experiments previously reported [1], taurine was found to be without effect on the conjugation efficiency when added to the homogenate system used. This was found to be due to the relatively high taurine content of the rat liver. However, when added to the

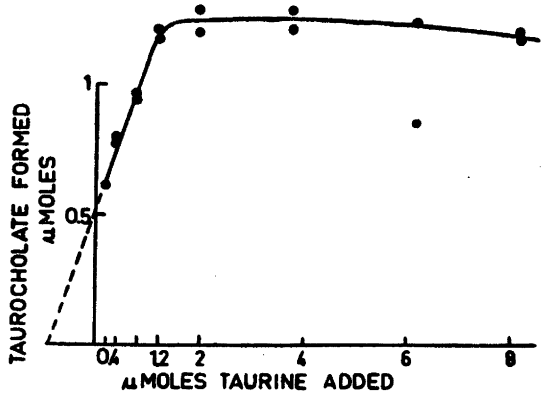

Fig. 8. Effect of taurine. Crude supernatant + the following additions: 2.5 μmole cholic acid, 0.2—0.4 μmole ^{35}S-taurine, 0—8 μmole unlabelled taurine. The conjugation was calculated from the amount of taurine added, the counting data and the calculated taurine content of the liver.

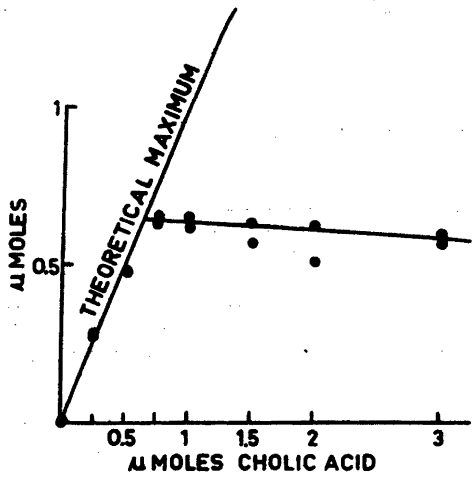

Fig. 9. Effect of cholic acid. Crude supernatant + the following additions: 0.4 μmole ^{35}S-taurine, 1.2 μmole ATP, 0—3 μmole cholic acid.

fortified system described in the present report, the formation of taurocholic acid was more than doubled (Fig. 8). The rise is almost linear up to the optimal taurine concentration (> 1.2 μmole added). Beyond this point, further addition had no effect.

Extrapolation of the curve gave a taurine content of approximately 0.8 μmole per 200 mg liver, *i. e.* 0.5 mg/g fresh liver. This is in good agreement with our earlier results on the normal taurine concentration in male rat liver [9].

In Fig. 9 is shown the effect of cholic acid on the formation of taurocholic acid in the presence of 0.4 μmole ^{35}S-labelled taurine. The conjugation of the cholic acid was quantitative until 90 % of the taurine was conjugated. Further addition had no effect. (Probably a still greater fraction of the taurine was conjugated, since the labelled taurine synthesized according to Eldjarn's procedure [10] has been shown to contain 3—4 % sulphate [11]). The form of the curve shows that the conjugation occurs almost quantitatively when equimolar amounts of taurine and cholic acid are added in small concentrations.

Stability of the enzyme system. The homogenate showed a rapid decrease of the activity when stored at 0° C. After 24 hours it was completely inactive. When stored for the same length of time in the frozen condition at —20° C, it had about 70 % of the activity left. A homogenate frozen and thawed again twice in rapid succession had still an activity of 85 %.

The isolated microsomes were completely unaffected by freezing, and they also showed full activity after 60 minutes' treatment with distilled water. No enzyme went into solution neither by this treatment, nor when the microsomes were frozen twice in distilled water.

Precipitation of the crude supernatant (III) or the isolated microsomes (IV) with acetone produced completely inactive powders, even when recombined

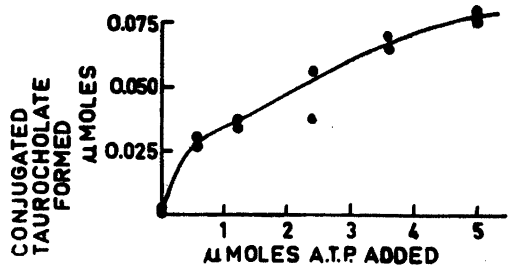

Fig. 10. Effect of ATP in the presence of boiled supernatant. Total volume 1.75 ml. 0.1 ml microsome suspension + 0.9 ml boiled supernatant from a 20 % homogenate kept in boiling water for five minutes and centrifuged free from precipitated proteins.

with fresh particle-free supernatant (V). The results were the same whether the homogenate or the microsomes were precipitated at 0° C or in the frozen condition.

On the other hand, when fresh microsomes from 1 ml homogenate were combined with acetone powder from 1—2 ml particle-free supernatant in the presence of ATP and DPN approximately 65 % of activity was restored. The possible influence of nicotinamide was not investigated in this preparation.

In Fig. 10 is shown that the boiled supernatant also was able partly to restore the activity of the microsomes when ATP was added. Thus in the presence of 5 μmole ATP approximately 20 % of the original activity was achieved. The possibility that fresh supernatant contaminated the isolated microsomes was excluded by an experiment where ATP was added also to isolated microsomes suspended in homogenizing medium. Only traces of activity could be detected. The ATP therefore had a greater effect when boiled supernatant was used than when fresh particle-free supernatant (V) was used. In the latter case ATP required addition of nicotinamide to give any effect (*cf.* Table 1). In the microsome+boiled supernatant system nicotinamide and DPN showed no effect.

DISCUSSION

Peptide bond synthesis has been studied by several workers in different systems.

Cohen and McGilvery [12] have published a series of works on the *p*-aminohippuric acid synthesis from *p*-aminobenzoic acid and glycine in rat liver slices and rat liver homogenate. They found the conjugation to be performed on the expence of phosphate bond energy by an enzyme system confined to the insoluble particles of the liver and kidney cell. Anaerobically the conjugation was strongly depressed if ATP was omitted from the reaction mixture. The conjugation was also depressed aerobically by substances known to inhibit oxidative processes, such as cyanide, azide and malonate.

Kielly and Schneider [13] have shown that the enzyme responsible for the conjugation of *p*-aminobenzoic acid is located in the mitochondria of the mouse liver cell, and Chantrenne [14] has succeeded in making an acetone extract from

mitochondria which catalyzes hippuric acid synthesis from benzoic acid and glycine when ATP, Mg^{++} and coenzyme A are added.

Schachter and Taggar [15] prepared benzoyl-coenzyme A and showed that this compound is active as a precursor to hippuric acid without any addition of ATP, Mg^{++} or coenzyme A. Their results strongly indicate that benzoyl-CoA is the "activated benzoic acid".

The biological synthesis of glutathion has been studied by Bloch [16,17]. Speck [18] has investigated the formation of glutamine from glutamate and ammonia. The participation of ATP and magnesium were also reported by these workers.

Coenzyme A has only been found to be necessary in the hippuric acid synthesis.

The results described in the present report show that the conjugation of taurine with cholic acid is very similar to the conjugation of glycine with benzoic acid or *p*-aminobenzoic acid, though the localization of the enzyme systems within the cell is different. The processes are all stimulated by factors concerned with oxygenation processes which procure energy for phosphate bond formation, and they are inhibited by substances interfering with these processes (cyanide).

Where probably most of the ATP-synthesizing enzymes have been omitted by combining fresh microsomes with boiled supernatant, no conjugation is achieved if ATP is omitted. In this case DPN and nicotinamide had no effect, whereas the effect of ATP was greater than when it was added alone to intact crude supernatant. At least one unknown factor from the supernatant must be necessary, as the isolated microsomes were inactive with all additions tried. This factor seems to withstand immersion in boiling water for five minutes and to follow the proteins when precipitated with acetone. In several isolation methods for coenzyme A, both boiling and precipitation with acetone are parts of the procedure [19]. Although we did not find any stimulating effect on the conjugation by the addition of a 6 % coenzyme A preparation, this compound may well be the unknown supernatant factor. Further work on this question is in progress.

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Preparation of 3β-Hydroxychol-5-enic Acid from Hyodesoxycholic Acid and Corresponding 24-^{14}C-labelled Acids. Bile Acids and Steroids 24

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The preparation of 3β-hydroxychol-5-enic acid-24-^{14}C *via* the dimethanesulphonylderivative of hyodesoxycholic acid-24-^{14}C is described. The preparation of the latter compound is also described.

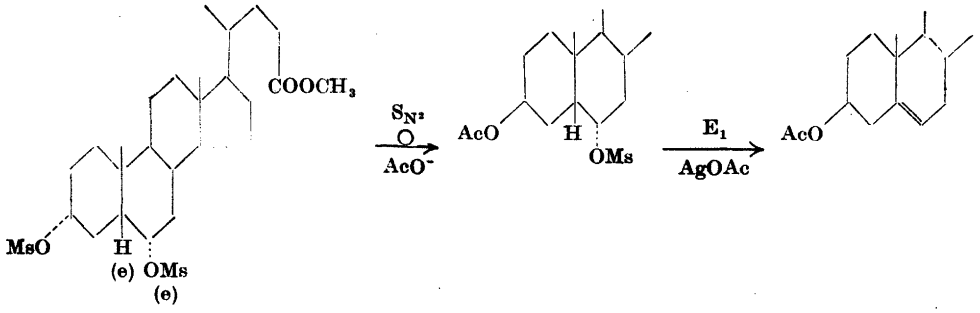
In connection with metabolic studies we needed some carbon labelled 3β-hydroxychol-5-enic acid. As the silver salt-bromine degradation [1] of the acetylated dibromide of this acid yielded mostly secondary products, the convenient nitrile-synthesis with labelled cyanide could not be utilized and other methods had to be sought. Methods for the preparation of 3β-hydroxychol-5-enic acid from hyodesoxycholic acid have recently been described. Yamataki and Ushizawa [2] treated methyl hyodesoxycholate with phosphorus oxychloride in pyridine and replaced the halogen in the 3β-chlorochol-5-enate so obtained with acetate. Shimitzu [3] transformed hyodesoxycholic acid into 3β,6β-dihydroxy *allo*cholanic acid according to Windaus [4] and the 6β-hydroxyl was then eliminated after protection of the 3β-hydroxyl through succinoylation.

We have worked out another procedure for this conversion based on earlier work by Lardon [5]. He found that a 3β,6β-dimethansulphonoxyandrostane derivative by treatment with silver acetate in boiling acetic acid yielded the corresponding 3β-acetoxyandrost-5-ene derivative, indicating an elimination reaction followed by acetolysis with retention of the configuration at C_3. However, the isomeric 3α,6β-dimethansulphonoxycoprostane derivative yielded under the same conditions the same 3β-acetoxycoprost-5-ene derivative, *i. e.* an indication that in this case the first reaction is an $S_{N'}$ reaction at C_3 with inversion followed by an elimination. Shoppee [6] has suggested that these observations indicate that the 6-methansulphonyl group in both these compounds are β-orientated. The *trans*-configuration would explain the more facile elimination in the former case, *cf.* also [7].

When we applied this method [5] to hyodesoxycholic acid (3α,6α-dihydroxycholanic acid), *i. e.* treated the dimesylate of methyl hyodesoxycholate with

silver acetate in boiling acetic acid, an acceptable yield (27 %) of 3β-acetoxy-cholen-5-ate could be isolated by chromatography of the reaction product. A considerable amount of a product with conjugated double bonds ($\varepsilon_{234} \sim 15\,000$) was also obtained but not characterized.

Concerning the mechanism of this reaction the conformation of A and B rings has to be considered. The occurrence of inversion of configuration at C_3 indicates that a normal S_{N^2} reaction with inversion has taken place before the elimination. Had the 5:6 double bond been formed first, a retention of configuration should have been expected. The reason for the less facile elimination of the 5-hydrogen and the 6-methane-sulphonoxy group in our case than in



Lardon's androstane derivative where the groups are presumable also *trans* might be that both groups are equatorial in hyodesoxycholic acid in relation to ring B whereas in the latter case they are both axial. The formation of products absorbing at 234 mμ indicates, that a double elimination also is taking place presumably with the formation of cholan-3,5-dienic acid.

The preparation of carboxy labelled hyodesoxycholic acid with the method of Bergström, Rottenberg and Voltz [1] is also described in the experimental part. From this material 3β-hydroxy-chol-5-enic acid-24-^{14}C has been prepared with the method outlined above.

EXPERIMENTAL

3α,6α-Diacetoxy norcholanylbromide. A mixture of hyodesoxycholic acid (10 g), pyridine (25 ml) and acetic anhydride (30 ml) was heated on the boiling water bath for one hour, then water (5 ml) was added slowly with cooling and the solution was then very slowly poured into about one litre of vigorously stirred ice water containing some ice. The acetylated acid then separated in a crystalline condition. The product was filtered and washed with cold water containing a little acetic acid until free from pyridine. After drying at room temperature the product melted at 95–98° C.

This product (10.5 g) was dissolved in 30 ml of ethanol and exactly neutralized with sodium hydroxide (phenolphthalein). A solution of silver nitrite (246 ml; 0.1 *M*) was then added slowly with shaking and the precipitated silver salt was isolated by filtration and washed with hot water. It was thoroughly dried over phosphorus pentoxide *in vacuo*. Yield 12.1 g.

Decarboxylation: About 200 ml of ethyl bromide was distilled from P_2O_5 onto a mixture of the finely prowdered silver salt (12.1 g) and silver acetate (3.5 g) in a system protected from the atmospheric moisture. Dry bromine (2.6 ml) was then slowly added to the refluxing suspension. The neutral reaction product was then isolated as described earlier. Yield 8.6 g.

The crude product was then chromatographed on alumina (50 g) in benzene-light petroleum (40°–60°) 4/6 (v/v). The material in the first 300 ml of eluate was combined and crystallized twice from light petroleum. Yield 3.2 g, M. p. 141–42° unchanged on further recrystallizations. (Found: C 63.41; H 8.36; Br 15.1. $C_{27}H_{43}O_4$ requires C 63.3; H 8.47; Br 15.6.)

Hyodesoxycholic acid-24-^{14}C. A mixture of 3α,6α-diacetoxy*nor*cholanylbromide (200 mg), $K^{14}CN$ (9.5 mg; 1 mC) and KOH (44.5 mg) in 5 ml of 80 % (v/v) ethanol/water was kept in a sealed tube in a water bath at 95° for 48 hours. The contents of the tube were then directly hydrolyzed in a steel tube at 140° for 7 hours after addition of 25 ml of 10 % (v/v) potassium hydroxide in 80 % ethanol. After evaporation of the ethanol the reaction mixture was acidified and extracted with ether. 68 mg of acidic products was extracted from the ether phase with dilute sodium hydroxide. An ether solution of the acidic products was treated with diazomethane and chromatographed on a column prepared from silicic acid (2 g) and Super-Cel (1 g) in methylene chloride. Methyl hyodesoxycholate was eluted with methylene chloride containing 2 % of methanol. Yield 40 mg, M. p. 75°.

Methyl 3α,6α-dimethanesulfonoxycholanate. Methyl hyodesoxycholate-24-^{14}C was dissolved in dry pyridine (1 ml) and methanesulpnonylchloride (0.15 ml) was added at 0°. The mixture was left at room temperature for 24 hours. The product was extracted with ether after dilution with water at 0° and the ether solution was washed successively with hydrochloric acid, water, carbonate and water. The dry product was a yellow amorphous foam (92 mg). (Found: S 11.0. Calc. for $C_{27}H_{46}O_8S_2$: S 11.4.)

Methyl 3β-acetoxychol-5-enate. A mixture of the dimethanesulfonylderivative of methyl hyodesoxycholanate (100 mg) and silver acetate (100 mg) was refluxed 2 hours in glacial acetic acid (3 ml). The mixture was evaporated to dryness *in vacuo* and the residue extracted with ether. This solution was washed with aqueous carbonate and water, dried over sodium sulphate. After evaporation to dryness, the residue (72 mg) was chromatographed on alumina (5 g). Each fraction = 30 ml.

1	Light petroleum/benzene	4:1	31.9 mg	oil, $\varepsilon_{234} \simeq 15\,000$ (M = 370)
2			62.4 »	
3			1.9 »	
4	»	2:1	18.0 »	crystalline, m. p. ~ 150°
5			17.5 »	
6	Benzene		16.5 »	
7			7.5 »	
8			2.3 »	
9	Benzene + 2 % MeOH		7.0 »	oil, S-containing
10			0.3 »	

The crystalline fractions (4–8) were combined and crystallized from methylene chloride-methanol. M. p. 154–55°, unchanged on further crystallization. (Found: C 75.0; H 9.8. Calc. for $C_{27}H_{42}O_4$: C 75.4; H 9.8.) The m. p. was not depressed by admixture of authentic methyl 3β-acetoxychol-5-enate (m. p. 155°). (Obtained from *Ciba Ltd*, Basel, through the courtesy of Dr. Wettstein.) The IR-spectrum and powder diffraction patterns of these preparations were also found to be identical. We are grateful to Prof. Stenhagen and Dr. Skogh at Uppsala University for these determinations.

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Instrumental Changes for Increasing the Precision of the Beckman Spectrophotometer, Model DU

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An account is given of instrumental changes made in the Beckman Spectrophotometer, model DU, for increasing the precision of the instrument and for facilitating its use and maintenance. Among the changes may be mentioned the use of a mains operated power supply, an external housing for the C-batteries, a constant voltage transformer stabilizing the filament voltage of the hydrogen lamp, the use of more stable tungsten lamps, insertion of high quality switches and desiccant boxes with glass windows.

When judging the reliability of an instrument, its accuracy and precision are to be considered. The closer the reading of the instrument is to the correct value, the better is the *accuracy* and the smaller is the *systematic error*. If, however, several measurements of the same quantity are performed, the individual readings differ in magnitude. The smaller these deviations are, the better is the *precision* and the smaller the *random error*.

In spectrophotometry there are several ways of increasing the accuracy and precision by arranging the *experimental factors* so as to give the most favourable condition for measurement (choosing correct wavelength, suitable absorbance of reference and sample solutions, cleaning of cuvettes, purifying of solvents, *etc.*). The aim of this article is to show that some *instrumental changes* of the Beckman spectrophotometer itself may increase the precision of the instrument.

Measurements in the Beckman spectrophotometer are performed by adjusting the dark current potentiometer, the slit or the sensitivity control and the density scale potentiometer, interrupted by the turning of the selector and of the shutter switches. From the foregoing it is evident that during these operations *all factors influencing previously made adjustments should remain unaltered*. Let us call this the *instrumental condition for precision*.

The operation of an instrument always means a certain strain on the operator resulting in operator's fatigue. This in turn results in decreased accuracy and precision of the measurement. *The more the performance of the measurement*

is facilitated, the less the operator's fatigue will be and the smaller the errors. Let us call this the *psychological condition for precision*.

The instrumental and psychological factors are often rather closely connected to each other. The separation of these factors made below should thus not be taken too literally.

INSTRUMENTAL CHANGES

The amplifier in the Beckman spectrophotometer, model DU, is a dc amplifier. A drawback of these amplifiers is their drift in amplification. Part of this drift can be eliminated by supplying the amplifier with constant voltages. An accumulator is used for the filament and bias voltages and built in dry cells for the phototube and anode voltages. In the visible range the tungsten lamp is also supplied from the accumulator, and as the drain of the lamp is considerable (5 A), the bias and filament voltages will gradually decrease thus requiring readjustment of the dark current and sensitivity controls. This can be avoided by providing separate accumulators for the instrument and the lamp.

Recently a voltage regulator, STAVOLT-A, has been developed specially for the Beckman DU *. It consists of three separate voltage regulators, one giving 2 volts dc for the filaments, one giving 6 volts dc for the bias and one giving 6 volts ac for the tungsten lamp. 115 volts unregulated ac is also supplied for the hydrogen lamp power supply. The use of separate circuits for the 2 and 6 volts dc supplies makes this regulator superior to previous commercial regulators for this purpose as the STAVOLT-A makes the settings of the sensitivity and dark current controls independent of each other, *i. e.* allows a "fine adjustment" of the slit width with the sensitivity control.

The 6 volts ac circuit of the STAVOLT-A may not be left unloaded when the regulator is in operation. To avoid burning the tungsten lamp when the hydrogen lamp is in use, a two-pole-two-position switch has been inserted in the STAVOLT-A. By means of this switch a resistance (three 5 ohms, 12 watt w.w. resistors in parallel) replaces the tungsten lamp when the hydrogen lamp is used. A 50 ohms resistor and a 0.05 μF capacitor in series have been connected in parallel with each pair of switch contacts to avoid spark generation resulting in oxidation.

The General Electric automobile lamps originally supplied for the Beckman instrument are not suited for ac. However, the Philips automobile lamp 6905-C works quite satisfactorily on ac and as it has the same socket as the General Electric bulb, they are directly interchangeable. The Philips lamp is not etched and may hence be used for the fluorescence adapter as well. As the Philips lamp gives a more intense light than the General Electric bulb, it may be used further down in UV, provided the visible stray light be filtered off.

The C-batteries of the Beckman instrument are located underneath the monochromator housing. In spite of the small drain, the batteries gradually become exhausted and the connections have to be altered. As this means turning the whole apparatus upside-down to and fro between each alteration

* Made by H. Struers Chemiske Laboratorium, Skindergade 38, Copenhagen, Denmark.

and checking, it is a cumbersome operation. *Cecchini and Eicher* [1] recently described the construction of an external C-battery supply, where the author has made some alterations [2]. Two such external C-battery supplies with Pertrix 1.5 E6 batteries have now been in daily use for 22 months at our institute without changes of batteries.

Finally the hydrogen lamp regulated power supply has been provided with a 2.8 volts, 10 amps. constant voltage transformer * controlling the filament current of the hydrogen lamp.

The electrical power sources thus being altered for the better still does not mean that the spectrophotometer will work satisfactorily. During measurements and between the different adjustments of the controls the selector and the shutter switches are switched to and fro. The zero position of the micro-amperemeter is often altered during these manipulations due to changed resistances between the switch contacts. An expedient is of course to substitute the switches by new ones.

Examination of the selector switch shows that it is a sixpole, four-place switch, whereas according to the circuit diagram a four-pole, four-place switch would suffice. The explanation is that four of the sections are paralleled, two by two, and these sections are only used as an on-off switch of the instrument. When turning the switch further from "Check" to "1" and "0.1" during measurements, the two paralleled pairs simply traverse short circuited contacts. Provided the contact resistances in these positions be changed during measurements, the instrument is supplied with altering voltages, *i. e.* different zero positions of the micro-amperemeter occur during switching.

The functions of switching the instrument on or off, and switching it between "Check", "1" and "0.1" were therefore separated. This was made by the inclusion of a standard two-pole, two-position on-off switch of good quality. It was mounted on the end of the monochromator housing just below the dark current control and the selector switch.

The original selector switch is of the Yaxley type. It has been substituted by an ELGE brush type instrument switch **. The switch used was a commercial one, a two-pole, seven-place, make-before-break switch.

A stop had to be soldered on the switch in order to change it to a three-place switch. All interconnections were then made on the switch and short connections soldered to the contacts by which the switch was to be connected to the instrument. After this the switch was washed first in carbon tetrachloride and then in grain alcohol. Thus all soldering paste, rosin and contaminations from handling the switch during soldering were removed from the contacts. Two contact strips, a four-pole for the on-off switch and a six-pole for the selector switch were mounted on the end of the monochromator housing below the on-off switch and the connections from the instrument and the switches were brought to these strips. In this way any contamination of the switch contacts with rosin during connecting it to the instrument could be avoided.

The new selector switch has a smaller angle between the steps than the old one. The old scale is thus not quite correct, but as the positions are brought

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* Raytheon, Model W 5855, made by Raytheon Manufacturing Co, Chicago, Ill., U.S.A.

** Made by ELGE, Vienna, Austria. A Painton Winkler Switch, Type AS/4P/3Rh/1B + K21 made by Painton Co.Ltd, Northampton, England may be even better.

down from four to three, this has not caused any trouble. On the other hand, by turning the switch one position too far to the left one cannot switch off the instrument by mistake, a blunder that previously led to a loss of at least 5 to 10 minutes in the measuring procedure.

A similar two-pole six-place ELGE brush switch with both sections paralleled has been inserted as a filament regulator switch in the hydrogen lamp power supply in order to obtain a more reliable operation. The resistance wire connected to the original switch was disconnected and transferred to the new one.

In spite of a thorough search the author has had no success in finding an instrument switch of the brush type small enough to use as a substitution for the original Yaxley type shutter switch. Discarded Beckman shutter switches were as a consequence rebuilt to brush type switches. The lever by which an arm of this switch moves the shutter to and fro is made of brass in its original design. As this lever was soon worn out, it was substituted by a replica in steel thus transferring the wear to the arm of the shutter switch, a detail that has to be exchanged at regular intervals.

SOME MINOR CHANGES FACILITATING THE OPERATOR'S WORK

The micro-amperemeter used as a zero instrument on the Beckman DU Spectrophotometer has a fine graduation. The reading is facilitated if a magnifying lens is placed over the scale. Another lens is tilted against it to concentrate the light from the window on the scale. This arrangement increases precision rather than accuracy. The parallax error of the system ought to be negligible.

As the first stage valve of the amplifier works as an electrometer valve it is important that no leakage currents occur in the amplifier. This is accomplished by keeping the air in the phototube housing dry with silica gel as drying agent in an desiccant box. Another desiccant box is placed in the monochromator housing. The condition of the desiccant may only be seen by opening the phototube or the monochromator housings. Desiccant boxes with glass windows for inspection of the condition of the silica gel have therefore been installed *. These boxes are suitably fastened to the housings from the *outside* for facilitating the change of silica gel.

For the insertion of these boxes, holes, 31 – 35 mm in diameter, have to be made in the housings, preferably with a radio chassis punch. Before the punching of the holes, the phototubes and amplifier valves have to be removed from the phototube housing, and the monochromator housing has to be removed as described in the instruction manual by changing the Helipot potentiometers. After punching all filings have to be carefully removed from the housings.

The disadvantage that this desiccant box has a smaller capacity than the original one in the monochromator housing is well counterbalanced by its advantages.

* Made by H. Struers Chemiske Laboratorium, Skindergade 38, Copenhagen, Denmark.

This work was undertaken during a comparative study on diffuse reflectance using different optical geometries where a high precision of the values was needed [3].

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Short Communications

Plant Growth Substances of the Cinnamic Acid Type

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As an extension of previous work on plant growth substances [1] a number of *trans*-cinnamic acids have been synthesized. Melting points and analytical data of these compounds are given in Table 1. Several of these acids have already been described in the literature but are reported here too as in some cases the experimental conditions were modified. In other cases new modifications or melting points, differing from those previously reported, were found. The experimental details will be given in a later communication.

The plant physiological properties of the compounds listed in Table 1 are now studied by Dr. Börje Åberg, who will publish his results in a separate paper. It may, however, already be mentioned that the well-known anti-auxin effect of *trans*-cinnamic acid is increased when chlorine is introduced into the aromatic nucleus.

This investigation of cinnamic acids as plant growth substances is also planned to comprise the *cis*-forms. The conditions for the preparation of *cis* isomers by ultraviolet irradiation of *trans* isomers are now under investigation as well as methods for the separation of the *cis* compounds from the reaction products.

Hydrocinnamic acids derived from the chlorocinnamic acids mentioned above should also be of some interest from a plant physiological point of view. As the chlorocinnamic acids are already available it should be advantageous if the hydrocinnamic acids could be prepared by reduction or hydrogenation. To avoid the simultaneous elimination of chlorine the experimental conditions must be properly chosen. Experiments are in progress with this object in view.

From a stereochemical point of view the α-methylhydrocinnamic acids attract particular interest due to the asymmetric α-carbon atom. It would be interesting to investigate the plant growth regulating properties of the optically active forms especially if it is possible to connect them sterically to the enantiomorphic α-aryloxyalkylcarboxylic acids which have previously been shown to possess stereochemical specificity as plant growth regulators [1,2].

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Table 1. Melting points (M. p.) and equivalent weights (Eq. wt.) of cinnamic acids.

Cinnamic acid	M. p. °C	Eq.wt.calc	Eq.wt.found
2-Chloro-	209—210	182.6	182.8
3- »	163—164.5	182.6	182.8
4- »	248—250	182.6	183.9
2,4-Dichloro-	232—234	217.1	217.9
3,4-	218—219	217.1	218.3
α-Methyl-2-chloro-	108—109.5	196.6	197.8
» 3- »	105.5—107	196.6	198.3
» 4- »	166—167	196.6	197.2
» 2,4-dichloro-	143—145.5	231.1	232.7
» 3,4- »	150—152	231.1	230.2

A Note on the Alleged Tetravalency of Antimony

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The black diamagnetic [1-3] double salts of tetravalent antimony: $(NH_4)_2SbBr_6$ and Rb_2SbBr_6 which K. A. Jensen [2] found to be cubic, are only pseudocubic. Using a 19 cm Bradley-Jay powder camera, and CoKα X-radiation, we have found that some of the lines which Jensen registered as singlets are in reality doublets. Powder photographs of $(NH_4)_2SbBr_6$, Rb_2SbBr_6 and Cs_2SbBr_6 can be indexed on the basis of a tetragonal lattice with $c/a = 1.43$. In an ideally cubic lattice $c/a = \sqrt{2} = 1.414$, hence the deviation from cubic symmetry, is about 1 % in the tetragonal salts. Rb_2SbCl_6 is strictly cubic. Its diagram, however, shows an abnormally rapid decrease in intensity with increase in glancing angle.

It has been found possible to prepare the following compounds which likewise have a cubic structure: $K_4FeSbCl_{12}$, $(NH_4)_4FeSbCl_{12}$, $Rb_4FeSbCl_{12}$ and $Cs_4FeSbCl_{12}$, all of which are yellow, and $(NH_4)_4InSbCl_{12}$ and $(NH)_4TlSbCl_{12}$, which are white or slightly violet.

After ageing for some years, the strictly cubic lattice of $(NH_4)_4FeSbCl_{12}$ is deformed into a tetragonal one with the axial ratio of $c/a = 1.40$. The atoms of this compound can be arranged in the space group $P4/mmm$ and the lattice change interpreted as being due to a transformation from a somewhat disordered arrangement of the Fe and Sb atoms to an ordered arrangement. With a primitive lattice, reflections are theoretically possible for any values of (h, k, l) but $h + k + l$ was found to be even for all the reflections observed except the one with the indices 102. A calculation of the intensities was carried out with the parameters chosen so that all metal-chlorine distances were set equal to 2.40 Å. The calculation showed that for such an arrangement the reflections with $(h+k+l)$ even would generally be of observable magnitude, while the 102 reflection would be the only one with an odd index sum strong enough to be found. It could be called a superlattice line of an I_4/mmm lattice.

It seems reasonable on the basis of the crystallographic data to ascribe the formula $Me_4Sb^{III}Sb^{V}X_{12}$ to the formally tetravalent black antimony compounds. In the tetragonal salts we assume an ordered arrangement of the trivalent and of the pentavalent antimony atoms, in Rb_2SbCl_6 a somewhat disordered arrangement.

Ephraim and Weinberg [4] have prepared a black cubic compound to which they ascribe the curious formula $9NH_4Cl$, $2FeCl_3$, $3SbCl_4$. Their compound is in reality a mixed crystal of a black salt, $Me_4Sb^{III}Sb^{V}X_{12}$ with a yellow salt of our new type $Me_4Fe^{III}Sb^{V}X_{12}$.

Jensen's theory [5] for the black colour of the tetravalent antimony compounds depicts a crystal of the salts as a single resonating system. This theory seems untenable in its present form since it assumes all antimony atoms in a crystal to be structurally equivalent, which in fact, they are not.

The following lattice constants have been found:

Cubic salts.

$K_4FeSbCl_{12}$	$a = 9.931$ Å
$(NH_4)_4FeSbCl_{12}$	$a = 10.01$ Å
$Rb_4FeSbCl_{12}$	$a = 10.18$ Å
$Cs_4FeSbCl_{12}$	$a = 10.22$ Å
$(NH_4)_4InSbCl_{12}$	$a = 10.09$ Å
$(NH_4)_4TlSbCl_{12}$	$a = 10.12$ Å
Rb_2SbCl_6	$a = 10.14$ Å

Tetragonal salts.

$(NH_4)_4FeSbCl_{12}$	$a = 7.098$ Å	$c = 9.915$ Å,	$c/a = 1.397$
$(NH_4)_2SbBr_6$	$a = 7.538$ Å	$c = 10.760$ Å,	$c/a = 1.427$
Rb_2SbBr_6	$a = 7.565$ Å	$c = 10.839$ Å,	$c/a = 1.433$
Cs_2SbBr_6	$a = 7.675$ Å	$c = 10.985$ Å,	$c/a = 1.431$
			$\sqrt{2} = 1.414$

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A Synthesis of 1,3-Dihydroxyacetone Convenient for ^{14}C-labelling

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A synthesis of $^{14}C-2$- and $^{14}C-1,3$-labelled dihydroxyacetone was previously described [1] but this method was rather tedious and did not give very high yield. In the following synthesis it was possible to obtain either ^{14}C-2- or ^{14}C-1-dihydroxyacetone by using $HOCH_2{}^{14}COOH$ or $HO{}^{14}CH_2COOH$ which had previously been prepared [2].

$$CH_3-COCl + HOCH_2-COOH \rightleftharpoons CH_3-COOCH_2-COOH \xrightarrow{SOCl_2} CH_3-COOCH_2-COCl \xrightarrow{CH_2N_2} CH_3-COOCH_2-CO-CHN_2 \xrightarrow{H_2SO_4} HOCH_2-CO-CH_2OH$$

The hydrolysis of acetoxydiazoacetone which was hard to control and easily gave rise to methylglyoxal and products of polymerisation has been followed attentively with paper chromatography.

Experimental. (1) *Acetylglycolic acid.* 5.0 g (0.066 mole) glycolic acid was refluxed with 10.0 g (0.127 mole) acetyl chloride for 45 minutes [3]. The excess of acetyl chloride was driven off on a water bath at reduced pressure. The residue was acetylglycolic acid, m. p. 66—68°

(2) *Acetylglycolic acid chloride.* The above acetylglycolic acid was immediately refluxed with 11.2 g (0.102 mole) thionyl chloride for 90 minutes. By fractional distillation of the solution at reduced pressure acetylglycolic acid chloride was obtained at 52—54°/12—14 mm Hg. Yield 8.0 g (0.059 mole) or 89 % from acetylglycolic acid.

(3) *Acetoxydiazoacetone.* 3.50 g (0.0256 mole) acetylglycolic acid chloride dissolved in 5 ml abs. ether was added drop by drop during 1 hour to 0.1 mole diazomethan dissolved in 150 ml abs. ether with mechanical stirring and cooling with ice-water. The solution was left for another hour at room temperature with continued stirring and was then evaporated on a water bath at reduced pressure. The residue was dissolved in 10 ml abs. ethanol.

(4) *Dihydroxyacetone.* From the alcoholic solution of acetoxydiazoacetone 3.0 ml were taken and diluted with 3 ml water and 3 ml of 4 *M* sulfuric acid was added dropwise. When about half the amount of sulfuric acid had been added the nitrogen evolution increased violently. The solution was cooled with ice-water until the reaction had decreased. The solution was kept at room temperature until only a very faint gas evolution remained and was then placed on a water bath (bath temp. 75 °) for 45 minutes. The colour of the solution was lemon yellow at first but grew deeper during the heating. After cooling with ice-water the solution was diluted with some water and neutralized with solid barium carbonate. The precipitated barium sulfate was removed by centrifugation and the filtrate was evaporated on a water bath (bath temp. about 35°) at reduced pressure. The concentrate was kept in a vacuum-desiccator with conc. sulfuric acid for 2 days. The solid glass-like residue was triturated with acetone and a fine white powder was formed. This substance reduced Fehling's solution in the cold and was identified as dihydroxyacetone with paper chromatography and m. p. 75—76°. The yield was 0.42 g (0.0047 mole), *i. e.* 61 % from acetylglycolic acid or 53 % from glycolic acid, respectively.

Attempts have been made to find a convenient method to synthesise ^{14}C-labelled dihydroxyacetone phosphoric acid. The following reaction series has been tested:

$$C_6H_5-CH_2OCH_2-COCl \xrightarrow{CH_2N_2} C_6H_5-CH_2OCH_2-COCHN_2 \xrightarrow{HO_2P(OCH_2-C_6H_5)_2} C_6H_5-CH_2OCH_2-COCH_2-OPO(OCH_2C_6H_5)_2 \xrightarrow{H_2} HOCH_2-CO-CH_2OPO_3H_2$$

These reactions gave a very low yield of dihydroxyacetone phosphoric acid especially owing to the difficulty of controlling the hydrogenation of the tribenzyl derivative. These experiments are still being continued.

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Vibrational Structure of a Spin-forbidden Band of Hexachlororheniate(IV)

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The absorption spectra of the complexes of elements in the second and third transition group can also be described by the crystal field theory, as will be shown later. These complexes are characterized by higher values of $(E_1 - E_2)$ than in the first transition group [1,2], as shown by Schmidt [3] in the special case of trivalent molybdenum compared to trivalent chromium. Another system with three d-electrons is tetravalent rhenium, of which the hexachloro and hexabromo complexes, $ReCl_6^{--}$ and $ReBr_6^{--}$, will be discussed here.

Maun and Davidson [4] studied solutions of rhenium(IV) complexes and found two narrow, weak bands in the red wavelength region. These are undoubtedly due to transitions from the ground-state $^4\Gamma_2$ to the states $^2\Gamma_3$, $^2\Gamma_4$ or $^2\Gamma_5$ of lower multiplicity, having the same strong crystal field electron configuration [2]. They are found at nearly the same wave number, $\sim$14 500 cm^{-1}, as the similar bands of molybdenum (III) [3] and chromium(III) [5]. The stronger bands, due to transitions to $^4\Gamma_5$ and $^4\Gamma_4$, are probably represented in $ReCl_6^{--}$ by the shoulder at 27 000 cm^{-1} found [6] at the very high "electron transfer" band in the ultraviolet. The K_2ReCl_6 was prepared according to Hurd and Reinders [7] and measured in 10 cm cells on a Cary spectrophotometer, using a 0.02 *M* solution in 10 *M* HCl. One of the two bands in the red shows a considerable fine-structure of equidistant bands with a half width $\sim$80 cm^{-1}. Fig. 1 shows the spectrum, and Table 1 the observed maxima of the first band group, giving the wave numbers ν_n and the molar extinction coefficients ε_n subtracted the constant background. The maxima are interpreted as a vibrational structure with a characteristic wave number $\nu_n = 150$ cm^{-1}, and values of Δn, the change in vibrational quantum number n, which are supported by the Boltzmann distribution of the levels at room temperature. If Gaussian analysis [8] is applied to the bands, the maxima of $\Delta n = -1$ and 2 are displaced to 13 880 and 14 350 cm^{-1}, respectively, giving more regular wave number differences.

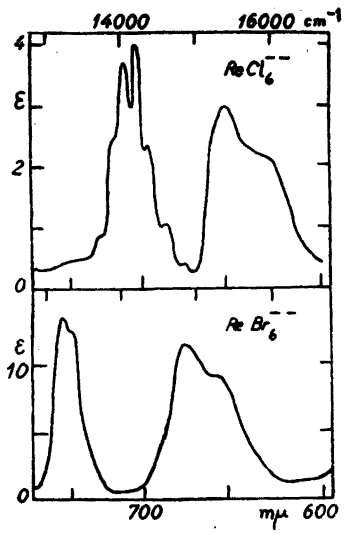

Fig. 1.

K_2ReBr_6 was prepared by boiling 0.5 g $KReO_4$ and 1 g KI with 20 ml 60 wt. % HBr for 30 minutes. The dark red crystals dissolved in 2 *M* HBr gave the spectrum shown in Fig. 1. The only trace of vibrational structure of the first band group is the distance 100 cm^{-1} between the two adjacent maxima. If the force constant discussed below is equal in $ReBr_6^{--}$ and $ReCl_6^{--}$, this change of γ_c is exactly predicted from the mass of the bromide ion compared to the chloride ion. The two red band groups are displaced $\sim$800 cm^{-1} towards lower wave numbers. Besides this, $ReBr_6^{--}$ gives a very complicated "electron

Table 1. The first band group of $ReCl_6^{--}$.

ν_n in cm^{-1}	ε_n	Δn
13 730	0.6	—2
13 900	2.2	—1
14 030	3.4	0
14 180	3.7	1
14 330	2.1	2
14 510	0.5	3
14 680	0.1	4

transfer" spectrum with shoulders and bands at 23 900, 26 400, 28 400, 30 800, 33 200 and 39 400 cm^{-1}, while the low shoulder at 20 900 cm^{-1} is probably due to crystal field transitions.

Schultz [9] attempted to obtain force constants from Raman spectra and infra-red measurements, but ν_c of $ReCl_6^{--}$ seems to be the first vibrational wave number, obtained from the visible spectrum of an octahedral complex. The most remarkable feature is its low value compared to ν_c of the tetrahedral MnO_4^- and linear UO_2^{++}, which are $\sim$750 cm^{-1}. The force constant k_1 is defined by $k_1 = 4\pi^2 mc^2 \nu_c^2$, m being the reduced mass of the oscillating ligand. In $ReCl_6^{--}$ k_1 is only $4.8 \cdot 10^4$ erg/cm^2, corresponding to a maximal deviation x from the equilibrium position of the ligand = 0.079 Å, 0.137 Å and 0.177 Å, respectively, for n = 0, 1 and 2 of the harmonic oscillator. Thus, the potential hole of the ligand must have quite a flat bottom, while the absolute depth must be considerable (at least 50 000 cm^{-1}). k_1 can only be relatively small, when the repulsing forces from the closed shells of Re^{+4} depend on a not very high power of the distance r from the nucleus, *i. e.* the bonding is distinctly electrostatic, while the complex is nevertheless robust [4].

The empirical fact [1] that the halfwidths of the ordinary broad bands of the transition group complexes are roughly proportional to the change of their wave number as a function of the crystal field strength, has been explained by Orgel [10] as an effect of the different equilibria positions of the ligands in the excited and the ground-state. Fig. 2 shows that simultaneous excitation of much higher vibrational states of the excited electronic state occurs when the ligand remains in the range of r, governed by the vibrations at room temperature (kT = 210 cm^{-1}) in the electronic ground-state. Since the halfwidths are nearly as large towards lower as towards higher wave numbers, and since no absorption limit is observed at even very low wave numbers of a given band, the most probable transitions must go to highly excited vibrational states. But if ν_c hardly can be larger than 200 cm^{-1}, this type of excitation necessitates values of $\triangle n \sim 10$ and of $x \sim 0.4$ Å, which seems improbable, even if somewhat lower values are possible, due to anharmonic parts of the potential curve. In the author's opinion, the broadening might also be connected with Heisenberg's uncertainty principle acting on the energy

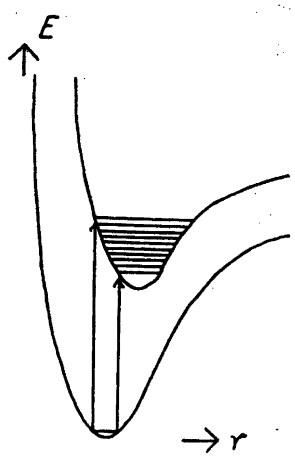

Fig. 2.

transfer from the excited state to the mechanical vibrations of the surrounding molecules, as pointed out in the case of uranyl nitrate by Stepanov [11]. If the energy is propagated with the velocity of 10^6 cm/sec. (somewhat more than of ordinary sound), it takes 10^{-14} sec. for the distance 1 Å, corresponding to an uncertainty of the wavenumber $\triangle \nu = 3\,300$ cm^{-1} (because $\triangle \nu \cdot \triangle t \cdot c \sim 1$). In this theory the narrow bands (*e. g.* of chromium(III) in the ruby or of europium(III) and terbium(III) salts) have larger uncertainties in time than 10^{-13} sec., corresponding to larger tendency of fluorescence. The explanation by Orgel [10] can be extended by the uncertainty principle by assuming the lifetime of the excited electronic state to be only a half period or less of vibration. When the ligand collides with the solvent molecules $\sim 10^{-14}$sec. after the excitation of the central ion, the energy is totally degraded to mechanical vibrations. This is only the case, when the equilibrium positions are different, corresponding to a different distribution of γ_5- and γ_3-electrons[10]. The difference between the equilibrium values of r needs only to be 2–5 %, which can be calculated by treating the crystal field perturbations as a part of the repulsing potental, *e. g.* proportional to r^{-6}.

Acknowledgments. I am very much indebted to Professor J. Bjerrum for his continued interest in the work, and to Dr. L. E. Orgel for the opportunity to see manuscripts previous

to the publication. Further, I thank Professor A. Tovborg Jensen for some grams of potassium perrhenate, and Miss Bodil Friis for assistance with the preparations.

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Mixed Monolayers of *Iso*dextropimaric and Normal Long Chain Fatty Acids

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In a previous study [1] it was found that the average area per molecule in fatty acid monolayers containing different amounts of *iso*dextropimaric acid (IdP) was higher than that expected if the mixtures behaved ideally. The work described in this paper, which is a continuation of the earlier investigation, has yielded data for mixed monolayers with high ratios of IdP to fatty acids. The data will be compared with theoretical deductions based on an assumed structure for the mixed monolayers.

The variation of the average area per molecule measured at a surface pressure of 1.5 dynes per cm with the composition of the IdP-fatty acid monolayers is shown in Fig. 1. The experimental details have been described in the earlier paper [1]. Except for the mixtures containing myristic acid (Curve C 14), which are of the expanded type at room temperatures, positive deviations from the ideal behaviour ($A_{mixt.} = x_1A_1 + x_2A_2$, the dotted lines) are evident in the curves. The expansions are greatest for the layers containing palmitic acid (Curve C 16) and decrease as the chain length of the fatty acid increases. At the same time, the maximal expansion shifts to owler fatty acid contents.

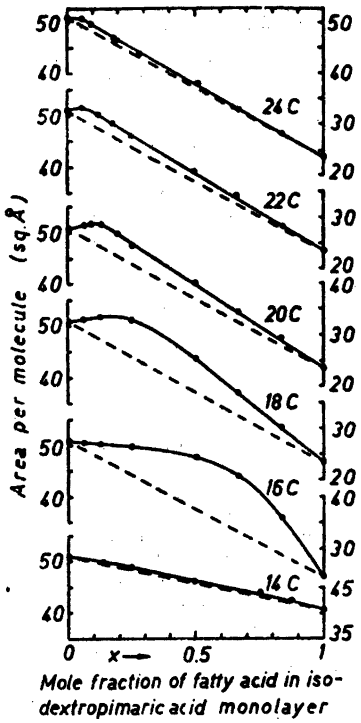

Fig. 1. Molecular areas of iso*dextropimaric acid monolayers containing normal fatty acids (C_{14}—C_{24}). Surface pressure, 1.5 dynes/cm. Substrate: dilute HCl (pH 3). 20° C.*

Owing to the different dimensions of the molecules of the components, the hydrocarbon part of the mixed monolayer may be considered as being composed of two layers, an upper layer formed by the parts of the flexible fatty acid chains that project above the IdP-molecules and a lower densely packed layer comprising bulky inflexible IdP-molecules and the lower parts of the

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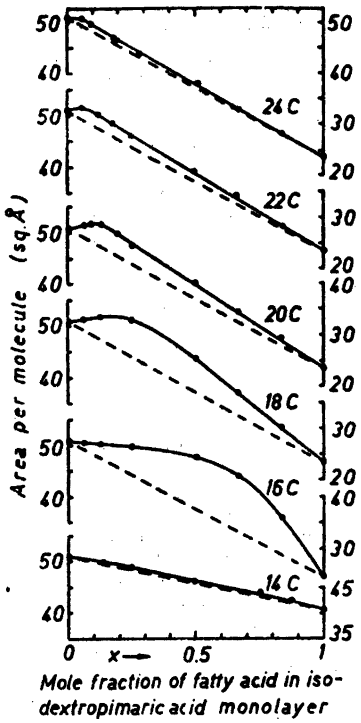

Fig. 1. Molecular areas of iso*dextropimaric acid monolayers containing normal fatty acids (C_{14}—C_{24}). Surface pressure, 1.5 dynes/cm. Substrate: dilute HCl (pH 3). 20° C.*

Owing to the different dimensions of the molecules of the components, the hydrocarbon part of the mixed monolayer may be considered as being composed of two layers, an upper layer formed by the parts of the flexible fatty acid chains that project above the IdP-molecules and a lower densely packed layer comprising bulky inflexible IdP-molecules and the lower parts of the

fatty acid chains. It is obvious that the average area per molecule in this "double" layer is primarily determined by the conditions prevailing in the densely packed lower layer, but it may be assumed to be mechanically influenced by the thermal agitation of the free chains in the upper layer. This latter expanding effect may be expected to increase with increasing fatty acid content when the latter is low since the projecting chains will not then affect each other's agitation owing to the large distances between them. At higher fatty acid contents, when the molecules are closely situated in the upper layer, the chains will interact and hence the agitation of the fatty acid chains will be more or less restricted. Owing to this the expansion of the layer will decrease with increasing fatty acid content. In view of the fact that the intermolecular forces between the hydrocarbon chains have a very short range, it seems probable that the maximum expansion occurs at the fatty acid content at which the agitating upper ends of the molecules first come into contact.

If the parts of the hydrocarbon chains in the upper layer are considered as swinging rods of small diameter (Fig. 2), the distance

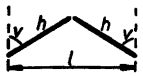

Fig. 2.

l between the axes of deflection of the two rods, the upper ends of which touch each other, is related to the length h of the rods by the equation

$$l = \sin v \times 2 h \qquad (1)$$

where v is the angle of deflection from the vertical. If the angle v is independent of h, we may write

$$dl/dh = \sin v \times 2 \qquad (2)$$

The mean distance l_m between the centres of two closest fatty acid molecules at maximum expansion of the monolayer can be calculated from the equation

$$l_m = \frac{4\sqrt{108}}{3} \cdot \left(\frac{1-x}{x} \cdot \bar{A}_{IdP} + \bar{A}_F\right)^{\frac{1}{2}} \qquad (3)$$

in which x denotes the mole fraction of fatty acid and $\bar{A}_F$ and $\bar{A}_{IdP}$ the respective partial molecular areas of the fatty acid and IdP. Values of l_m based on the experimental data are given in Table 1.

Table 1.

Number of C-atoms in fatty acid	x (at maximal expansion)	$\bar{A}_{IdP}$ (Å²)	$\bar{A}_F$ (Å²)	l_m from Eq. (3)	h_F from Eq. (4)
16	0.60	59.5	37.0	9.4	8.4
18	0.32	58.0	31.5	13.4	11.0
20	0.17	56.0	24.0	18.5	13.5
22	0.10	53.5	23.5	24.1	16.0
24	0.07	52.5	23.5	28.9	18.6

The maximal length of a fatty acid molecule is found on the basis of bond lengths and atomic radii to be $(1.27 \times n_c + 4.0)$ Å were n_c denotes the number of carbon atoms in the molecule. The length of the IdP-molecule [2] is 15.8 Å. The length of the part of the fatty acid molecule projecting above the IdP-molecules in hence

$$h_F = (1.27 \times n_c - 11.9) \text{ Å} \qquad (4)$$

When l_m is plotted against h_F for the different fatty acid-IdP-layers, the following linear equation is obtained:

$$l_m = 2\ h_F - 8 \text{ Å} \qquad (5)$$

The slope of this line is 2, in agreement with Eq. (2) when $\sin v = 1$, *i. e.* when the angle of deflection is 90°. (Calculations based on data recorded at a surface pressure of 10 dynes per cm give the same slope.)

By substituting $l_m = 0$ in Eq. (5), the value $h_F = 4$ Å is found which gives the length of the ineffective part of the chain forming the upper layer that does not contribute to the sideward deflection of the chain. This length probably corresponds to the curved part of the deflected hydrocarbon chain.

It seems that a method based on this theory of folding of fatty acid molecules around other molecules can be developed which will permit a relatively accurate determination of the dimensions of molecules in mixed monolayers.

I wish to express my gratitude to Professor Per Ekwall, Ph. D., the Head of the Institute, for allowing me to use the facilities of the Institute and for discussions. I am grateful to Professor Einar Stenhagen of the Institute of Medical Chemistry of Uppsala University for samples of the three higher fatty acids and to

Dr. C. G. Harris of Hercules Powder Company, Wilmington, USA, for the sample of *iso*dextropimaric acid.

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Sialic Acid in Pseudomyxomatous Gels

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It is well known that glycoproteins occur in considerable amounts in the contents of certain types of ovarian cysts. Little recent work, however, has been published on the nature of these glycoproteins, except for the extensively studied blood-group substances, which were shown to occur in pseudomucinous cyst fluids by Morgan and van Heyningen [1]. The carbohydrate component of the blood-group substances has been shown to consist of glucosamine, galactosamine, galactose, and fucose [2-4]. Hiyama [5] has isolated the hexosamines and galactose from the gelatinous 'paramucin' of an ovarian cyst, noting that the material gave the 'direct Ehrlich' reaction, *i. e.* it gave a violet colour when heated with Ehrlich's *p*-dimethylaminobenzaldehyde reagent without previous treatment with alkali. Jensen [6] has isolated a product from a few pseudomucinous cyst fluids thought to be hyaluronic acid. (I have not been able to find this substance in the same type of cysts.)

In a study on the protein and glycoprotein components of a great number of ovarian cyst contents and their relation to the histological type of the cysts, I have found that pseudomyxomatous gels differ not only physically but also chemically from ordinary pseudomucin. An obvious difference is the much higher content of sialic acid in the gels (about 10 % compared with 1–2 %, as determined colorimetrically). Sialic acid has now been isolated in crystalline form from this material, and by chemical analysis and X-ray powder diagrams is shown to be identical with the sialic acid prepared by Blix *et al.*[7] from the submaxillary mucin of sheep.

The pseudomyxomatous material, which forms a water-insoluble gel, was washed with water and broken up in a Turmix blendor. After several days in ethanol, when the gel had shrunk considerably, it was ground in a mortar and after further treatment with ethanol and ether, dried in a desiccator.

The results of analysis of a typical preparation from a cyst-gel are given in Table 1.

Table 1.

Nitrogen (Micro-Kjeldahl)	10.2 %
Glucosamine-HCl *	5.8 »
Galactosamine-HCl *	5.7 »
Galactose **	3.6 »
Mannose **	0.6 »
Fucose **	2.1 »
Sialic acid ***	11.8 »
Ester-sulphate	0.1 »
Ash	3.7 »

* Chromatographic separation of the hexosamines by Gardell's method [8].

** Vasseur's modification of the Tillmanns-Philippi orcinol reaction [9] and a quantitative paper chromatographic method.

*** Colour reactions with Bial's and Ehrlich's reagents [10].

From this material, thoroughly freed from inorganic matter by treatment with very dilute hydrochloric acid, sialic acid was prepared according to the principles set out by Blix [11].

10 g of the dry powder was suspended in 200 ml of water, and heated for one hour on a boiling water-bath. The suspension was centrifuged, and the supernatant filtered and freeze-dried. The small amount of material obtained was extracted with methanol, and the solvent

Fig. 1. X-ray powder diagram of sialic acid from an ovarian cyst-gel.

Dr. C. G. Harris of Hercules Powder Company, Wilmington, USA, for the sample of *iso*dextropimaric acid.

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evaporated *in vacuo*. The residue was dissolved in methanol and a little water. Ether was added, and the amorphous precipitates, which formed regularly, were discarded. Crystalline deposits were obtained in a few days. These were purified by recrystallization from a water-methanol mixture.

Some analytical data of the crystalline material are given in Table 2.

Table 2.

Nitrogen	4.38 %
Acetyl	14.96 »
Methoxyl	0
Decomposition-point	185—187° (uncorr.)
Optical rotation	$[\alpha]_D^{23} = -32° \pm 2°$

The X-ray diffraction pattern is seen in Fig. 1. It is identical with that of sialic acid isolated from sheep's submaxillary mucin.

The same sialic acid was isolated from the pseudomyxomatous contents of two other cysts, and from the peritoneal gel of a pseudomyxoma peritonei also present in one of the cases. (The contents of a mucocele appendicis in the last case had the same composition as the other gels. Isolation of sialic acid was not attempted because of lack of material.)

A detailed report of this work will be published elsewhere.

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A New Method for Peroxide Determination

SVEN ARRHENIUS

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In a previous paper [1] vanillin dissolved in 70 % sulphuric acid was shown to be a qualitative reagent on peroxides. In trying to apply this test for quantitative analysis we found that with regard to colour production, hydrogen peroxide differs from the tertiary butyl peroxides $(H_3C)_3C-OOH$ and $(H_3C)_3C-OO-C(CH_3)_3$. Hydrogen peroxide (Fig. 1) gives a green colour, only slightly dependent on the added amount of peroxide. Thus this colour reaction can not be used as a quantitative reagent. Moreover, the colour is not sufficiently different from that of aged solutions of protocathecuic aldehyde (demethylated vanillin) or piperonal (vanillin -2 H) in 70 % sulphuric acid.

The tertiary butyl peroxides also colours vanillin—sulphuric acid green, but after a few hours the colours shift over violet into deep blue (Fig. 1). The blue reaction products (called "vanillin-blue" in this paper) precipitate on diluting the sulphuric acid solution with ice water, preferably with at least five volumes. The precipitate dissolves completely without impairement of the colour in concentrated acids, like sulphuric, phosphoric, acetic or formic. Hydrochloric acid should be avoided as evolution of chlorine was observed. Organic bases and alkali destroy vanillin-blue. The precipitate was washed with ice water until the pH of the water was 5, and dried *in vacuo* over $CaCl_2$. In standing at room temperature, vanillin-blue changes colour and solubility.

The formation of vanillin-blue increases with temperature. As the peroxides and vanillin-blue itself, however, decompose on heating, it will be an optimum for the reaction. According to preliminary experiments, this optimum is about 37° C. There are also many practical reasons to use this temperature, and all data given here are obtained by heating the samples at 37° C. The formation of vanillin-blue is slower at higher concentrations. For practical reasons the reaction was stopped after 20 hours. Consequently, the dosage curves will not be straight lines but curved downwards.

evaporated *in vacuo*. The residue was dissolved in methanol and a little water. Ether was added, and the amorphous precipitates, which formed regularly, were discarded. Crystalline deposits were obtained in a few days. These were purified by recrystallization from a water-methanol mixture.

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A New Method for Peroxide Determination

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In a previous paper [1] vanillin dissolved in 70 % sulphuric acid was shown to be a qualitative reagent on peroxides. In trying to apply this test for quantitative analysis we found that with regard to colour production, hydrogen peroxide differs from the tertiary butyl peroxides $(H_3C)_3C-OOH$ and $(H_3C)_3C-OO-C(CH_3)_3$. Hydrogen peroxide (Fig. 1) gives a green colour, only slightly dependent on the added amount of peroxide. Thus this colour reaction can not be used as a quantitative reagent. Moreover, the colour is not sufficiently different from that of aged solutions of protocathecuic aldehyde (demethylated vanillin) or piperonal (vanillin -2 H) in 70 % sulphuric acid.

The tertiary butyl peroxides also colours vanillin—sulphuric acid green, but after a few hours the colours shift over violet into deep blue (Fig. 1). The blue reaction products (called "vanillin-blue" in this paper) precipitate on diluting the sulphuric acid solution with ice water, preferably with at least five volumes. The precipitate dissolves completely without impairement of the colour in concentrated acids, like sulphuric, phosphoric, acetic or formic. Hydrochloric acid should be avoided as evolution of chlorine was observed. Organic bases and alkali destroy vanillin-blue. The precipitate was washed with ice water until the pH of the water was 5, and dried *in vacuo* over $CaCl_2$. In standing at room temperature, vanillin-blue changes colour and solubility.

The formation of vanillin-blue increases with temperature. As the peroxides and vanillin-blue itself, however, decompose on heating, it will be an optimum for the reaction. According to preliminary experiments, this optimum is about 37° C. There are also many practical reasons to use this temperature, and all data given here are obtained by heating the samples at 37° C. The formation of vanillin-blue is slower at higher concentrations. For practical reasons the reaction was stopped after 20 hours. Consequently, the dosage curves will not be straight lines but curved downwards.

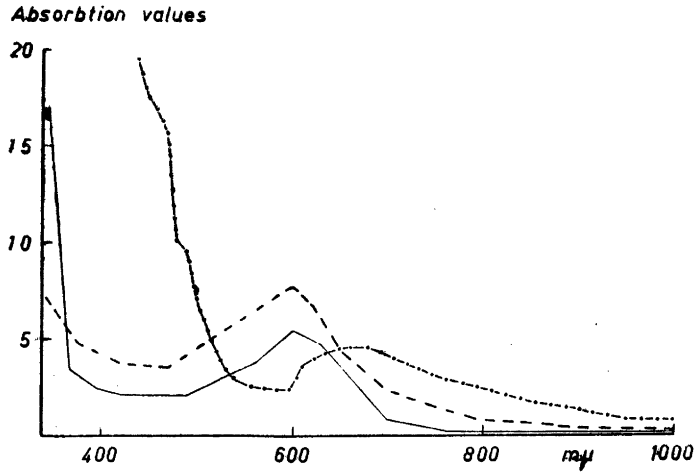

Fig. 1. Extinction curves for vanillin-sulphuric acid treated with: a. Hydrogen peroxide (1×10^{-4} M) — · — · — · —. b. t-BuOOH (1.7×10^{-5} M), diluted with ethanol ————. c. t-BuOOH, precipitated, and dissolved in formic acid — — — — —

The most accurate method of analysis for *t*-butyl peroxides, based on formation of vanillin-blue, will be to filter off the precipitate, wash and dissolve it in formic acid, and determine the extinction at 600 mμ. By this treatment coloured impurities disturbing the measurements are removed. Already the pure reagent itself strongly colours the supernatant solution brilliant red (abs.-max. at 500 mμ). The particle size of the vanillin-blue precipitate, however, is so that about 30 % goes through glass filter G 4. In using a finer filter good results were obtained, but the time for filtering will be too long for practical use. The old trick of clotting colloidal precipitates of this kind by heating fails here, since vanillin-blue is heat-sensitive.

Compounds showing extinction at 600 mμ are rather uncommon, and hence direct determination of the reaction product will be convenient. Generally the colouration is so strong that the solutions have to be diluted before extinctiometry. A dilution with 70 % sulphuric acid shifts the absorbtion maximum over to 560 mμ, and the dosage curve (Fig. 2) is strongly bent. In using ethanol for dilution, the absorbtion maximum (Fig. 1) remains at 600 mμ, and the dosage curve is straighter. With both kinds of dilution the colour remains constant for a couple of hours at room temperature, or for the sulphuric acid dilutions for a day in the ice-chest.

Method. To 1 ml, or less, of an aqueous solution containing *t*-butyl hydroperoxides or di-*t*-butyl peroxides, add 10 ml of a solution of 15.2 mg vanillin (10^{-2} *M*) in 70 % (by volume) sulphuric acid. After 20 h in a water bath at 37° C, dilute with 20 ml ethanol, and measure the extinction in a Klett-Summerson photometer using filter 60. At this dilution the method covers the concentration range 10^{-5} to 10^{-6} moles (1—100 μg).

Discussion

The dosage curves approache straight lines when smaller amounts of peroxides than 10^{-6} moles are added to the vanillin-sulphuric acid. For analytical purposes it will be better to use this method at higher concentrations only for orientation. The great deviations from the dosage curves may be ascribed to errors at the dilution, which at 10^{-5} moles was 1:100. Another error may be due to the dilution of the sulphuric acid in mixing it with the sample. The formation of vanillin-blue is restricted to concentrations between 60 % and 80 % with an optimum at 70 %. If larger amounts than 1 ml are to be added to 10 ml vanillin-sulphuric acid, the acid must be more concentrated, so as to obtain 70 % after mixing.

In the optimal region (10^{-8} to 10^{-6} moles added peroxide), the extinction at 600 mμ is $(31.5 \pm 0.7) \times 10^8$ units/mole, for dilu-

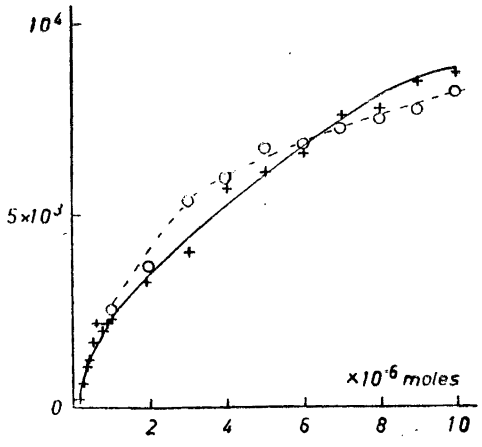

Fig. 2. Dosage curves for vanillin-blue:
a. Diluted with 70 % sulphuric acid o——o
b. Diluted with 96 % ethanol +——+

tion with ethanol, and $(28.4 \pm 0.9) \times 10^8$ units/mole, for sulphuric acid dilution. The advantage in diluting with ethanol is the maximal absorption at 600 mμ. The interference with other coloured substances formed, *i. e.*, the red substance in the supernatant liquid will be less in ethanol. On the other hand the alcoholic solutions are less stable. A point to be noted in the practical application of the method is that both an aged vanillin-sulphuric acid solution and a freshly prepared one will give the same dosage curve.

For the use of vanillin-blue as a peroxide reagent, it is unnecessary to know its formula or the reaction mechanism. The amount of vanillin exceeds greatly that of the peroxides (ranging from 1:10 to 1:10^4). It is doubtful whether the vanillin-blue formed and precipitated has a constant composition. However, an analysis was made, giving C 68.2, H 5.46, and O 24.3 (1 % ash).

Campbell and Coppinger [2] added *t*-butyl hydroperoxide to 2,6-di-*t*-butyl-*p*-cresol, and obtained a substance described as 1-methyl - 1 - *t* - butylperoxy - 3,5 - di - *t* - butylcyclohexadienone-4. With this type of peroxide (which have an absorption maximum at 234 mμ) vanillin-blue seems to have little in common. We exclude a compound with the *t*-BuOO-group directly attached to the aromatic ring as such compounds will not be stable. In view of the small amounts of peroxide needed for the formation of colour and the unknown behaviour of less stable peroxides such as the methylhydroperoxide, no formula for vanillin-blue is advanced.

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4s-Electrons in the First Transition Group Complexes

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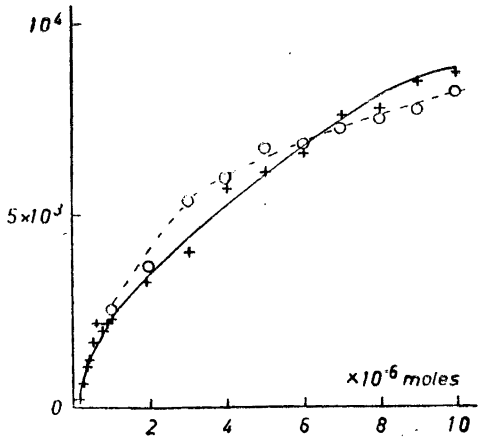
When the crystal field theory is applied [1,2] to the absorption spectra of hexaaquo ions and other simple complexes of the first transition group, small discrepancies (~ 5 %) occur between the calculated and observed wavenumbers of the band maxima. Mainly based on magnetic evidence, Owen [3] has interpreted these effects by ~ 20 % intermixing of σ-electrons from the ligands with the γ_3-electrons (the highest energy state of d-electrons in an octahedral complex while the other is γ_5). In the present note attention will be drawn to the electron configuration $3d^{n-1}$ 4s, which in the free, divalent ion is situated [4] ~ 50 000 cm^{-1} over the groundstate, due to $3d^n$.

Since these two electron configurations have the same parity (the sum of the l-values is even) they can directly intermix. The author [5] even maintains that the crystal field induces an intermixing with $3d^{n-1}4p$ and electron transfer states, explaining the observed transitions which would else be forbidden by Laporte's rule. The former interaction will *inter alia* depress the energy of the levels with the same Γ_n as the lowest level of the excited $3d^{n-1}$ 4s configuration. Thus, in d^3-systems, the levels $^4\Gamma_4$ will be depressed by the groundstate $\gamma_5^2\gamma_1$ of $3d^24s$. Now, Mr. C. E. Schäffer has kindly pointed out to me that the observed energy difference, $^4\Gamma_4 - {}^4\Gamma_5$, between the two strong bands of chromium (III) complexes decreases by increasing crystal field strength, while it should [6] be slightly increasing or constant, if no interaction appeared. Also V^{++} shows this effect if $^4\Gamma_5$ is placed at 12 200 cm^{-1}, contrary to Owen [3].



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Since these two electron configurations have the same parity (the sum of the l-values is even) they can directly intermix. The author [5] even maintains that the crystal field induces an intermixing with $3d^{n-1}4p$ and electron transfer states, explaining the observed transitions which would else be forbidden by Laporte's rule. The former interaction will *inter alia* depress the energy of the levels with the same Γ_n as the lowest level of the excited $3d^{n-1}$ 4s configuration. Thus, in d^3-systems, the levels $^4\Gamma_4$ will be depressed by the groundstate $\gamma_5^2\gamma_1$ of $3d^24s$. Now, Mr. C. E. Schäffer has kindly pointed out to me that the observed energy difference, $^4\Gamma_4 - {}^4\Gamma_5$, between the two strong bands of chromium (III) complexes decreases by increasing crystal field strength, while it should [6] be slightly increasing or constant, if no interaction appeared. Also V^{++} shows this effect if $^4\Gamma_5$ is placed at 12 200 cm^{-1}, contrary to Owen [3].

In d^5-systems as Mn^{++}, Orgel[2] discussed a systematical depression of ^4G and other excited terms in the hexaaquo ion, compared with the free ion [4]. This might also be explained by interactions especially with the low $^4\Gamma_3$ and $^4\Gamma_4$ of $3d^44s$. With the Cary spectrophotometer a very broad band ($\delta \sim 1\,500$ cm^{-1}) is observed of 2 M $MnSO_4$ in H_2O at 32 600 cm^{-1}. This is undoubtedly due to $^4\Gamma_4$, and the band[1] at 29 700 cm^{-1} to $^4\Gamma_3$, as pointed out by Orgel[2]. At 38 500 and at 40 600 cm^{-1}, two bands are observed, probably due to $^4\Gamma_4$ and $^4\Gamma_5$ of the term ^4F, which should be displaced upwards by perturbations [2]. $^4\Gamma_2$ can be assigned to one of the two shoulders observed at 35 900 and 37 000 cm^{-1}. The three latter levels show definitely the decrease in energy of ^4F, due to interactions with states of other electron configurations, *e. g.* $3d^44s$. All the quartet levels of $3d^5$ in Mn^{++} seem now detected.

In d^8-systems as Ni^{++}, the lowest levels of $3d^74s(\gamma_5^6\gamma_3\gamma_1)$ are $^3\Gamma_3$ and $^1\Gamma_3$. The latter level will depress the lowest singlet level [7] of nickel(II) complexes. Without this interaction the transition $^3\Gamma_2 - {}^1\Gamma_3$ would correspond to the weak band [2,7] at 18 350 cm^{-1} of $Ni(H_2O)_6^{++}$. But the "extra" band at 15 400 cm^{-1} has been assigned [5] to this transition. In a paper to be published the energy of $^1\Gamma_3$ in a wide range of nickel(II) complexes will be studied. Table 1 shows the observed maxima in cm^{-1} of complexes, which are not all of cubic symmetry (*e. g.* the ethylenediaminetetraacetate or the solutions in concentrated acids). The parentheses indicate cases of very strong intermixing, where the distinction between $^1\Gamma_3$ and the triplet state has no physical significance [8]. This results in nearly equal intensities and halfwidths, while $^1\Gamma_3$ ordinarily is weak and narrow.

While the band [7] should be quite constant $\sim 18\,000$ cm^{-1} as an intermixing of ^1D and ^1G found by Shenstone [9], the observed values decrease considerably with increased crystal field strength. In tetragonal complexes the interaction will be even more pronounced. Thus, the energy difference, necessary to make a planar complex diamagnetic, will be rather 11 000 than 18 000 cm^{-1} (*cf.* eq. 29 of Ref. 7). This agrees better with the observed tendency of changing groundstate.

The ethanol solvate of nickel(II)bis-(acetylacetonate), which probably is octahedral [10], has a shoulder at 13 000 cm^{-1} besides the stronger triplet bands at 9 100 and 15 550 cm^{-1}. If nickel nitrate in 60 % glycerol is cooled by liquid air, a sharp band in the far red can be distinguished in a spectroscope, corresponding to a later place in the Table. The non-diagonal elements [8] in the matrices between ^1D and ^3F, due to (L, S) coupling, are half the smallest distances between the two bands, when the levels are crossing, ~ 800 cm^{-1} in the table. If the non-diagonal elements between $3d^n$ and $3d^{n-1}4s$ are of the order of magnitude of 10 000 cm^{-1}, then the intermixing in squares of the wavefunctions will be about 4 %, and the energy decreases of the lowest levels $\sim 2\,000$ cm^{-1}, agreeing well with the observed effects.

Table 1.

	$^1\Gamma_3(D)$	$^3\Gamma_5(F)$	$^3\Gamma_4(F)$	$^3\Gamma_4(P)$
free	14 000	0	0	16 900
H_2SO_4	14 800	—	12 200	23 350
H_3PO_4	14 900	—	13 150	24 500
$(H_2O)_6$	(13 500)	8 500	(15 400)	25 300
enta	12 700	10 100	17 000	26 200
glycine$_3$	13 100	10 100	16 600	27 600
$(NH_3)_6$	13 150	10 750	17 500	28 200
en$_3$	12 400	11 200	18 350	29 000
$\alpha\alpha'$-dip$_3$	(11 500)	(12 650)	19 200	—
o-phen$_3$	(11 550)	(12 700)	19 300	—

Transitions between the configurations $3d^n$ and $3d^{n-1}4s$ can next be sought for in the absorption spectra. Orgel [2] pointed out that copper(I) complexes do not show such bands in the wavenumber range 21 900 – 26 300 cm^{-1}, where levels of ^3D and ^1D of $3d^9$ 4s are distributed in the free ion [4]. Solutions of $Cu(NH_3)_2^+$ in 0.2 M NH_4ClO_4 and 1 M NH_3 show on the Cary absorption above 35 000 cm^{-1}. If this is identified as these transitions, giving not much higher intensities than the usual crystal field spectra, it is seen that the 4s-electron has considerably higher energy in the crystal field than in the free ion. This is formally connected with the different values [11] of the crystal field parameter B_0 in different configurations. Orgel [12] has reviewed the "electron transfer" spectra and agrees with Dainton that some bands with $\varepsilon \sim 100$ of divalent ions in the ultraviolet are due to transfer of electrons from the central ion to the ligands, the opposite way of the ordinary bands (with $\varepsilon \sim 5\,000$) found in oxidizing metal ions such as Fe^{+3}, Cu^{+2}, Ir^{+4}, Pu^{+4}, *etc.* The former type of band, observed in V^{++} at 33 000 cm^{-1} and in Cr^{++} at 40 000 cm^{-1} may be ascribed to the $3d^{n-1}4s$ states. Since ^4F and ^5F of these configurations are

situated at 44 000 and 49 000 cm^{-1} in the free ions [4], the crystal field splittings of the excited terms must here be considerable in order to explain the low wavenumbers observed. It might be argued that ^7S of $3d^5 4s$ in Fe^{++} at 30 000 cm^{-1} should give even lower wavenumbers. But for more than five d-electrons, the highest multiplicity of $3d^{n-1}4s$ gives spin-forbidden bands, and first ^5S at 41 000 and ^5G at 63 000 cm^{-1} will give ordinary intensities in Fe^{++}, where a band [12] is observed $\sim$40 000 cm^{-1}.

Orgel [2] and Owen [3] investigate the possibility of covalent bonding, *i. e.* molecular orbitals being occupied by electrons from both central ion and ligands. This is undoubtedly the case of ligands with considerable electron affinity, as CO, CN^-, NO^+, aromatic amines, PCl_3, trialkylphosphines, but it is not easily decided in the case of ordinary ligands (H_2O, NH_3, *etc.*) where the most conspicuous effect [2,3] on the absorption spectra is only an increased energy difference ($E_1 - E_2$) between γ_3- and γ_5-electrons, analogous to the crystal field influence. It is interesting that ($E_1 - E_2$) is nearly constant $\sim$20 000 cm^{-1} in trivalent hexaaquo ions, while it is $\sim$10 000 cm^{-1} in divalent ions, and here decreasing [1] with the atomic number as implied from the theory [13]. This can only be explained by considerably smaller distances to the effective negative charges of the ligands of the trivalent ions than in the divalent ions, if the covalent hypothesis is not accepted. It must be remarked that the parameters in the crystal field model of Ilse and Hartmann [13] have no quantitative physical significance. *E. g.*, the hydrogen-like 3d wavefunction with the effective charge $Z = 4$ has its maximum at a distance 1.18 Å from the nucleus, while the radius of Ti^{+3} is assumed to be 0.8 Å. Some problems related to effective quantum numbers will be discussed elsewhere [14]. The transitions between different configurations, $5f^n \rightarrow 5f^{n-1}6d$ in the actinide ions, are also known from absorption spectra [15].

A valuable implication from the theory of molecular orbitals [2,3] is that the strong electron transfer bands are due to transitions from the odd γ_4-states. Hartmann [16] pointed out that the series of energy of the molecular orbitals in an octahedral complex should be:

$$\gamma_1, \gamma_4, \gamma_3, \gamma_5(d), \gamma_3(d), \gamma_1(s), \gamma_4(p) \ldots$$

If the lowest γ_3 had a much higher energy than the lowest γ_4, weak bands would be found in the electron transfer spectra at lower wavenumbers than the strong bands.

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Dihydro-thionaphthene-2- and -3-carboxylic Acids

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The acids have been prepared in connection with current work on optically active plant growth substances. They are easily obtained by hydrogenation of the corresponding thionaphthene-carboxylic acids using sodium amalgam.

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Dihydro-thionaphthene-2- and -3-carboxylic Acids

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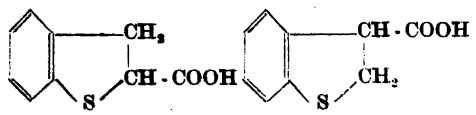
The acids have been prepared in connection with current work on optically active plant growth substances. They are easily obtained by hydrogenation of the corresponding thionaphthene-carboxylic acids using sodium amalgam.

The biological properties are being investigated by several scientists. The 3-acid (II) possesses auxin activity while the 2-acid (I) seems to be an anti-auxin.

The 2-acid is readily resolved into the enanthiomorphs using brucine and cinchonine. The rotatory power is rather high (*ca.* 365° in absolute ethanol); this is no doubt due to the cyclic structure and the presence of a sulphur atom linked to the asymmetric carbon. The resolution will be described in detail elsewhere.

The alkaloid salts of the 3-acid have a rather low tendency to crystallise and the resolution of this acid has not yet been completed.



(I) (II)

Experimental. Dihydro-thionaphthene-2-carboxylic acid (I). The corresponding thionaphthene-2-carboxylic acid was prepared by metalation of thionaphthene with *n*-butyllithium and subsequent treatment with carbon dioxide; the method was given by Shirley and Cameron [1] and independently by Gronowitz [2].

Sodium hydroxide (25 g) was dissolved in 400 ml water and 18.0 g (0.1 mole) thionaphthene-2-carboxylic acid was added. A sparingly soluble sodium salt separated. Sodium amalgam, prepared from 6.5 g sodium and 260 g mercury, was added with stirring during 20 minutes. As the sodium salt of the dihydro acid is readily soluble, the precipitated salt goes into solution as the reaction proceeds; it was completely dissolved after 45 minutes. The solution was stirred for another 45 minutes and left to stand with the amalgam overnight. The mercury was then separated and the solution neutralised and filtered with carbon. The acid was precipitated with sulphuric acid in excess. After drying it was recrystallised four times, alternatingly from dilute formic acid and benzene. Glistening plates or flat needles with m.p. 112—113°. (Found: C 60.01; H 4.48; S 17.70; equiv. wt. 180.4. $C_9H_8O_2S$ requires C 59.98; H 4.47; S 17.78; equiv. wt. 180.2.)

Dextrorotatory acid. Racemic acid was converted to brucine salt, which was recrystallised four times from 50 % aqueous methanol; the activity remained constant after two recrystallisations. The acid was liberated and recrystallised twice from ligroine (b. p. 85—110°). Plates or flat needles with m. p. 94.5—95°. (Equiv. wt. found 180.5; calc. 180.2. $[\alpha]_D^{25} = +365.4°$ in absolute ethanol.)

Levorotatory acid. The acid liberated from the first mother liquor of the brucine salt was converted to cinchonine salt, which was recrystallised four times from dilute ethanol; the activity remained constant after two recrystallisations. The acid was liberated and recrystallised as described above. Plates or flat needles with m. p. 94.5—95°. (Equiv. wt. found 180.3; calc. 180.2 $[\alpha]_D^{25} = -365.8°$ in absolute ethanol.)

Dihydro - thionaphthene - 3 - carboxylic acid (II). The corresponding thionaphthene-3-carboxylic acid was prepared from 3-bromothionaphthene according to Komppa and Weckman [3]. The yield could be considerably improved by performing the Grignardation in the presence of an equivalent amount (excess not necessary) of ethyl iodide according to Crook and Davies [4]. It is also convenient to dilute the Grignard solution with dry benzene before the treatment with carbon dioxide.

22 g (0.122 mole) thionaphthene-3-carboxylic acid were dissolved in 500 ml 2 *N* sodium hydroxide solution. Sodium amalgam, prepared from 6.5 g sodium and 200 g mercury, was added with stirring in the course of one hour. The stirring was continued for six hours and the solution was left to stand over-night. It could be noticed that the reaction proceeded slower than for the 2-isomeride.

The solution was separated from the mercury, the acid was precipitated with sulphuric acid in excess, decolourised with charcoal and recrystallised from cyclohexane. Long, glistening needles with m. p. 99.5—100.5°. (Found: C 59.83; H 4.40; S 17.77, 17.63; equiv. wt. 180.5. $C_9H_8O_2S$ requires C 59.98; H 4.47; S 17.78; equiv. wt. 180.2.)

The author is indebted to Mr. *S. Gronowitz* for an ample supply of thionaphthene-2-carboxylic acid and to Mr. *J. Lindh* for help with part of the experimental work.

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A Convenient Synthesis of DL-*homo*Methionine (5-Methylthio-*nor*valine)

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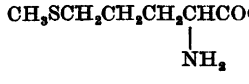
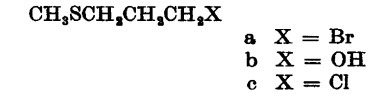
A three-step synthesis of *homo*methionine (I) in an over-all yield of 70 % is described. It proceeds from dimethyl formamidomalonate which is smoothly allylated to (X). UV- and peroxide-catalyzed addition of methyl mercaptan affords in quantitative yield the ester (XI), which is hydrolyzed by acid to give *homo*methionine. The latter is characterized as its N-formyl- and N-benzoyl-derivatives. The phenylthiohydantoin (IX) is prepared upon reaction of (I) with phenyl *iso*thiocyanate.

A similar sequence of reactions is described with the usual, but less conveniently prepared, *diethyl* formamidomalonate as a starting material. Alkylation experiments have demonstrated methylthiopropyl halides to be of little use in the present case.

In the course of studies on the phytochemistry of naturally occurring *iso*thiocyanates a need arose for an authentic specimen of DL-*homo*methionine (5-methylthio*nor*valine) (I).



I



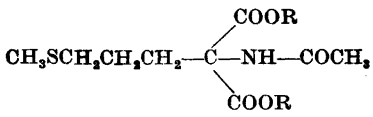
II

This amino acid, which has not been encountered in Nature, was first synthesized in the laboratory of du Vigneaud [1] by a fairly circumstantial procedure, not specifically designed for the synthesis of *homo*methionine. Later Gaudry *et al.*[2] reported rather low yields in the synthesis of (I) from γ-bromopropylhydantoin and methyl mercaptan, followed by hydrolysis with alkali.

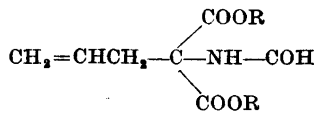
We preferred to build up (I) by alkylation of a suitable α-acylamidoacid ester possessing a reactive α-hydrogen atom. Our first efforts, however, were directed towards the synthesis of the previously unknown 3-methylthiopropyl bromide (IIa) by the addition of methyl mercaptan to allyl bromide. Although reaction took place it was unfortunately accompanied by undesirable side reactions, and this observation led us to reinvestigate the addition of methyl mercaptan to allyl alcohol previously reported by Kaneko [3]. With slight modi-

fications the addition reaction gave *quantitative* yields of (IIb), thus exceeding those of formally analogous reactions, *e. g.* the addition of benzyl mercaptan to allyl alcohol, reported by Brown, Jones and Pinder [4] to proceed in 51—70 % yield, dependent on the type of catalyst. The exchange of the hydroxy-grouping of (IIb) with chlorine was effected with thionyl chloride as described by Kirner [5,6], resulting in a 67% yield of (IIc). An alternative and equally satisfactory way of preparing the latter was found in the preferential reaction at the 1-bromo position of the commercially available 1-bromo-3-chloropropane with sodium methylmercaptide. Further reaction products obtained from the differently prepared chlorides proved identical, a fact which served to confirm that the addition had taken place as desired (contrary to Markownikoff's rule).

Several attempts to alkylate sodium ethyl acetamidocyanoacetate with (IIc) gave crystalline (III) in a maximum yield of only 11 %, large amounts of an intractable brown oil being simultanously formed. Cautious alkaline hydrolysis of (III) afforded the corresponding free acid (IV).



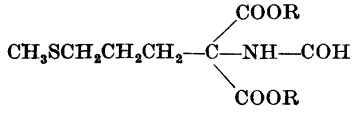
III R = C_2H_5
IV R = H



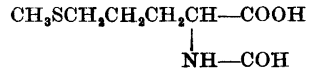
V R = C_2H_5
X R = CH_3

No better results attended numerous attempts in which (IIc) or (IIa) were condensed with sodium ethyl formamidomalonate [7] in stead of the cyanoacetic acid derivative. Again, considerable amounts of dark-coloured, secondary products appeared, possibly due to the formation of sulphonium-type by-products. The unsatisfactory results were rather unexpected in view of the reported smooth condensation of the lower homologous 2-methylthioethyl chloride with sodium ethyl formamidomalonate, an essential step in two methionine syntheses [8,9]. Consequently, further attempts to utilize methylthiopropyl halides were abandoned and other approaches considered.

Ethyl allylformamidomalonate (V) was readily obtained by allylation of sodium ethyl formamidomalonate in almost quantitative yield as described by Koštíř and Král [10]. Addition of methyl mercaptan to (V) under the usual conditions (UV-light, benzoyl peroxide and mercuric acetate) afforded a quantitative yield of the crystalline adduct (VI).



VI R = C_2H_5
XI R = CH_3

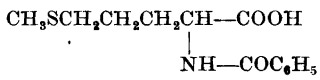


VII

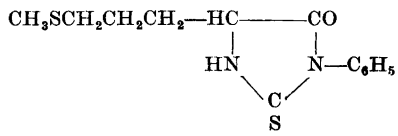
Upon treatment of the latter with the calculated amount of methanolic potassium hydroxide at reflux temperature N-formyl*homo*methionine (VII) resulted; no special effort was made to isolate formamidomethylthiopropyl-

malonic acid which is presumed to be formed as an unstable intermediate in the reaction. The formyl derivative (m. p. 122-123°) was previously used [1] for the characterization of *homo*methionine; our pure preparation was found to melt at 128-129° with some previous sintering.

Acid hydrolysis of either (VI) or (VII) yielded *homo*methionine hydrochloride which was in turn transformed into the free amino acid (I) by passing through an anion exchange resin (Amberlite IR-4B) in its hydroxyl form. The high melting point reported [1] (272-274°) (corr.)) could not be reproduced for our specimen, which heated in capillary tubes melted at *ca.* 235°, whereas rapid heating on the Fisher-John's block gave the value 247-248°, both accompanied by extensive decomposition (Ref.[2] lists m. p. 254-255° (dec.)). For further characterization *homo*methionine was transformed into its N-benzoyl derivative (VIII) and phenylthiohydantoin (IX), the latter prepared by the general method of Edman [11].



VIII



IX

While the present work was in progress a paper by Hellmann and Lingens [12] appeared in which the advantage of using *methyl* formamidomalonate instead of the usual ethyl ester in amino acid syntheses was pointed out. We could confirm the convenient synthesis of the methyl ester in almost quantitative yield as stated, and consequently carried through the above sequence of reactions also in the methyl series. The allylation, addition and hydrolysis all proceeded in nearly quantitative yields. Therefore, this route to *homo*methionine from easily accessible starting materials in an over-all yield of *ca.* 70 % represents a facile and convenient synthesis. With obvious alterations the same sequence of reactions can be utilized for the preparation of a series of analogous S-alkyl homologues of α-amino acids, *e. g. homo*ethionine.

The paperchromatographic behaviour of *homo*methionine was studied in two solvent systems. The R_F-values, together with those of some natural α-amino acids, partly appearing in the same range, are listed in Table 1.

EXPERIMENTAL

All melting points are uncorrected and determined in capillary tubes unless otherwise stated. Those below 80° were determined in a water bath, the remaining ones in an electrically heated block.

3-Methylthiopropanol (IIb). Since Kaneko's work was accessible only in abstract form [3] with few experimental details we were obliged to reinvestigate the optimal conditions for the addition reaction. In a round-bottomed quartz flask were placed 35 ml (0.52 mole) of freshly distilled allyl alcohol together with 100 mg of benzoyl peroxide and 200 mg of mercuric acetate. The solution was cooled and an ampoule containing 15.6 g (0.33 mole) of methyl mercaptan introduced and crushed beneath the surface by means of a glass rod. The flask was tightly stoppered and placed in front of a quartz-mercury lamp for

Table 1. R_F-*Values of* homo*methionine and various other amino acids determined on Whatman paper No. 1 by the descending technique.*

Amino acid	*n*-Butanol/Acetic acid/Water 4:1:3	Pyridine/Amyl alcohol/Water 35:35:30
Glycine	0.15	0.03
Valine	0.47	0.14
Leucine	0.63	0.24
*iso*Leucine	0.61	0.23
Methionine	0.49	0.17
*homo*Methionine	0.54	0.22
Phenylalanine	0.57	0.25
Tyrosine	0.43	0.20

12—16 hours while being cooled by a stream of cold water. After filtration, distillation *in vacuo* afforded 33.2 g (94 %, calc. on methyl mercaptan) of pure 3-methylthiopropanol, b. p. 89—90° at 13 mm. Ref.[5] reports b. p. 93—94° at 17 mm.

3-Methylthiopropyl chloride (IIc). By the modified method[6] of Kirner[5] the above alcohol (84.4 g) was transformed into the corresponding chloride (66.6 g), *i. e.* 67 % yield. B. p. 73.5—74.5° at 28 mm. Reported[5]: b. p. 71.2° at 29 mm.

The same chloride was obtained from 1-bromo-3-chloropropane in the following way. To a well-cooled solution of 3.80 g (0.165 mole) of sodium in 80 ml of anhydrous ethanol was added 7.83 g (0.163 mole) of methyl mercaptan; the dihalide (25.8 g; 0.154 mole) was then introduced, resulting in an immediate reaction. The calculated amount of sodium bromide was removed by filtration and the filtrate fractionated *in vacuo* yielding 16.3 g (85 %) of 3-methylthiopropyl chloride, b. p. 63—64° at 19 mm.

N-Acetyl-2-cyano-2-(3'-methylthiopropyl)-glycine ethyl ester (III). To a solution of 2.30 g (0.10 mole) of sodium in 50 ml of thoroughly dried ethanol were added 17.02 g (0.10 mole) of ethyl acetamidocyanoacetate (commercially obtained from the Sterling-Winthrop Co.), followed by 13.00 g (0.104 mole) of 3-methylthiopropyl chloride. The mixture was refluxed for 20 hours, protected from atmospheric moisture by a calcium chloride tube. The dark-brown mixture was then poured on to 250 g of crushed ice when a brown oil separated, together with smaller amounts of a crystalline product. The latter was isolated by filtration, washed with ice-water and dried, 2.80 g (11 %). After recrystallization from dilute ethanol (Norit) and then twice from ethyl acetate and petroleum ether, colourless needles were obtained, m. p. 80.5—81.5°. (Found: C 51.24; H 6.87; N 10.70; S 12.28. Calc. for $C_{11}H_{18}O_3N_2S$: C 51.15; H 7.02; N 10.85; S 12.42). Experiments conducted with stirring, shorter reaction times, and with the exclusion of air proceeded essentially as described with no improvements of the yield.

N-Acetyl-2-cyano-(3'-methylthiopropyl)-glycine (IV). A solution of 0.79 g (3.05 mmoles) of the above ester in 3.45 ml of 0.89 *N* methanolic KOH and 4 ml of water was left at room temperature for 72 hours. The methanol was removed *in vacuo* and the aqueous solution freed of unreacted ester by extraction with chloroform. The well-cooled aqueous solution was acidified to pH 2 and the colourless precipitate (0.65 g, 93 %) collected by filtration. The product was rapidly recrystallized from water, care being taken not to raise the temperature above 50° where vigorous decarboxylation sets in. The dense, shiny crystals melted with decomposition at 130—131°. (Found: C 47.17; H 6.26; N 11.94; S 14.02. Calc. for $C_9H_{14}O_3N_2S$: C 46.93; H 6.13; N 12.17; S 13.92).

Diethyl allylformamidomalonate (V). This substance was prepared in 96 % yield essentially as described[10]. M. p. 64—65°; Ref.[10] reports m. p. 64°.

Diethyl (3-methylthiopropyl)-formamidomalonate (VI). A cooled solution of 14.0 g (0.057 mole) of the allyl ester (V) in 35 ml of absolute ethanol, containing 30 mg of benzoyl peroxide and 150 mg of mercuric acetate was placed in a quartz flask. 2.95 g (0.061 mole) of methyl mercaptan were added and the cooled and tightly stoppered reaction vessel

was exposed to ultraviolet light for 18 hours. When the reaction was over the contents were brought into solution by moderate heating on the steam bath and a slight, insoluble residue removed by filtration. The filtrate was concentrated *in vacuo* to a colourless oil, care being taken to keep the bath temperature below 45°. The oil was treated with ice-water when it crystallized to give 15.13 g (91 %) of (VI), sufficiently pure for further use. An analytical sample was obtained as thin, shiny plates after three recrystallizations from aqueous ethanol, m. p. 64°, the same as that of the starting material. A mixture, however, melted at 48—50°. (Found: C 49.32; H 7.11; N 4.98; S 11.24. Calc. for $C_{13}H_{21}O_5NS$: C 49.46; H 7.27; N 4.81; S 11.00).

Dimethyl formamidomalonate. This ester was prepared as described [11]. In order to obtain the best yields (*ca.* 95 %) we found it necessary to employ zinc powder activated by short exposure to hydrochloric acid.

Dimethyl allylformamidomalonate (X). Dimethyl formamidomalonate (56.0 g) was alkylated with allyl bromide in dry methanol solution, following the procedure used for the preparation of the ethyl ester. In a preliminary experiment where ethanol was used as a solvent transesterification to the ethyl ester occurred. The yield was 55.0 g (80 %) of crystalline ester, considerably more soluble in water than the ethyl ester above. An analytical specimen was prepared by two recrystallizations from water, m. p. 75—76°. (Found: C 50.30; H 5.97; N 6.65. Calc. for $C_9H_{13}O_5N$: C 50.23; H 6.09; N 6.51).

Dimethyl (3-methylthiopropyl)-formamidomalonate (XI). The addition of methyl mercaptan to (X) was performed as described above in the ethyl series, but with methanol as the solvent. From 19.6 g (0.091 mole) of (X) and 7.20 g (0.15 mole) of methyl mercaptan, 22.9 g (96 %) of almost pure (XI) was obtained. A sample for analysis was recrystallized twice from water, m. p. 72.5°. (Found: C 45.45; H 6.32; N 5.34. Calc. for $C_{10}H_{17}O_5NS$: C 45.60; H 6.51; N 5.32).

N-Formyl-DL-homo*methionine* (VII). The esters, (VI) or (XI), were refluxed for 30 minutes with exactly two equivalents of 0.89 *N* methanolic KOH. Water was added to dissolve the sodium salt and the methanol removed *in vacuo*. The cooled solution was acidified to pH 2 with 6 *N* HCl, accompanied by a vigorous evolution of CO_2. The clear solution was left at 0° overnight when a 55 % yield of (VII) separated. An additional amount was obtained after evaporation of the filtrate to dryness and repeated extractions of the residue with hot ethyl acetate. An analytical specimen was isolated as small rhombic plates from ethyl acetate. M. p. 128—129° after slight sintering from *ca.* 122°. Ref.[1] reports the m. p. 122—123°. (Found: C 43.87; H 6.78; N 7.39. Calc. for $C_7H_{13}O_3NS$: C 43.97; H 6.85; N 7.33).

DL-homo*Methionine* (I). The formyl derivative (VII) or the esters, (VI) or (XI), were hydrolyzed to the amino acid (I). Generally, the compound in question was refluxed with a large excess of constant boiling HCl for several hours. In one experiment, in which HBr was used, a concomitant cleavage of the C—S bond appeared to occur as indicated by the evolution of methyl mercaptan. Excess hydrochloric acid was removed by repeated evaporations with freshly added portions of water. The dilute solution of *homo*methionine hydrochloride was slowly passed through a column of Amberlite IR-4B in its hydroxyl-form (*ca.* 20 g of resin per 5 g of amino acid). The chloride-free eluate was concentrated to dryness *in vacuo*. The residue was dissolved in a small amount of hot water; cautious addition of ethanol resulted in the separation of *homo*methionine in beautiful hexagonal plates. After being kept for 24 hours at 0° the crystals were collected by filtration and dried over P_2O_5 at 100° *in vacuo*. The yield was 85—90 %. The amino acid decomposed at *ca.* 235° in capillary tubes. Upon rapid heating on the Fisher-John's block the m. p. 247—248° (dec.) was observed. (Lit. values: 272—274° (corr.) [1], 254—255° (dec.) [2]). (Found: C 44.23; H 7.95; N 8.75; S 19.50. Calc. for $C_6H_{13}O_2NS$: C 44.16; H 8.03; N 8.58; S 19.64).

N-Benzoyl-DL-homo*methionine* (VIII). To a cooled and stirred solution of 163.2 mg of (I) in 0.85 ml of 1.20 *N* NaOH and 5 ml of water, were simultaneously added 0.14 ml of freshly distilled benzoyl chloride and 0.90 ml of 1.20 *N* NaOH. After stirring at room temperature for an hour the solution was acidified to pH 1—2; the resulting precipitate was filtered off and thoroughly washed with petroleum ether. In this way the benzoyl derivative (245.8 mg) was obtained in 92 % yield. It was recrystallized twice from aqueous ethanol for analysis as colourless needles, m p. 135° (Fisher-John's block). (Found: C 58.38; H 6.18; N 5.10. Calc. for $C_{13}H_{17}O_3NS$: C 58.40; H 6.41; N 5.24).

3-Phenyl-5-(3'-methylthiopropyl)-2-thiohydantoin (IX). A solution of 450 mg of *homo*methionine in 20 ml of 60 % pyridine was brought to pH 8.6 by adding 1 *N* NaOH (0.12 ml). 1.5 ml of phenyl *iso*thiocyanate were added and the solution kept between pH 8.4 – 8.6 by gradual addition of 1 *N* NaOH (2.72 ml used; calc. 2.76 ml). After 2 hours the reaction was over and excess reagent was removed by extraction with benzene. The aqueous phase was covered with 20 ml of ethyl acetate and acidified to pH 2 with 4 *N* HCl. The organic phase was separated, dried and taken to dryness, leaving an oil which was dissolved in 5 ml of glacial acetic acid saturated with dry HCl. The solution was left overnight and taken to dryness at 30 – 40° *in vacuo*, when 670 mg (87 %) of the crystalline thiohydantoin resulted. Two recrystallizations from ethanol afforded an analytical sample with m. p. 111°. (Found: C 55.56; H 5.65; N 10.00; S 22.98. Calc. for $C_{13}H_{16}ON_2S_2$: C 55.68; H 5.75; N 9.99; S 22.87).

Microanalyses were performed in this laboratory by Mr. W. Egger and Mr. P. Hansen. A valuable gift of dimethyl malonate from *A/S Grindstedværket* is greatly acknowledged. The authors are thankful to Dr. P. Edman, Lund, Sweden for helpful suggestions and to Mr. K. Lauber for the preparation of the thiohydantoin. The work is part of investigations supported by *Carlsbergfondet (The Carlsberg Foundation)*.

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Magnetokinetic Studies of the Reaction of Hydrogen Peroxide with Haemoglobin in Dithionite Solutions

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The changes of magnetic susceptibility which accompany the reaction have been recorded. The results have been interpreted in relation to the simultaneous changes of spectral absorption, and show that a transient compound of haemoglobin with distinctive spectral absorption and relatively low magnetic susceptibility is formed. A tentative value of 4 500 $\times$ 10^{-6} cgs emu has been obtained for the molar paramagnetic susceptibility of the compound. It cannot be decided at the moment whether the transient compound is ferric or ferrous.

The addition of hydrogen peroxide to a neutral or slightly alkaline solution of haemoglobin containing an excess of sodium dithionite ($Na_2S_2O_4$) results in the destruction of some of the haemoglobin and the formation of small amounts of choleglobin. In spectrokinetic studies of this reaction, Dalziel and O'Brien [1-3] detected the formation of a transient intermediate compound of haemoglobin. They showed that within a few seconds of the addition of hydrogen peroxide the spectrum of haemoglobin, λ_{max} 430 mμ and 555 mμ, was completely displaced by an entirely different absorption curve, with distinct and symmetrical maxima at 417, 545, and 582 mμ. This new spectrum persisted, unchanged except for a slow decrease of intensity, for periods of seconds or minutes, according to the reactant concentrations and the pH, and then gradually gave way to a haemoglobin spectrum of reduced intensity, with an additional small peak at 630 mμ due to choleglobin. The life of the transient spectrum increased with the initial peroxide concentration and was reduced by the presence of catalase. The spectrum differed significantly from that of known haemoglobin derivatives, including methaemoglobin-hydrogen peroxide, but was quantitatively reproduced over quite a wide range of reactant concentrations. The experimental facts suggested that the spectrum might characterise a hitherto unrecognised peroxide com-

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pound of haemoglobin; the formation of "haemoglobin-hydrogen peroxide" as an intermediate in such reactions had been postulated by Lemberg, Legge and Lockwood [4,5]. Conclusive evidence of the nature of the compound, or even of the state of oxidation of the haem iron, could not be obtained, however, in this peculiar reaction mixture, and the possibility that the transient spectrum was due to a mixture of methaemoglobin-hydrogen peroxide and some unknown degradation product could not be excluded.

Theorell and Ehrenberg [6] determined the molar magnetic susceptibilities of the unstable peroxide compounds of peroxidase, catalase and metmyoglobin by means of simultaneous measurements of light absorption and susceptibility. Their apparatus [7] was specially designed for rapid measurements on dilute solutions. It seemed worth while to attempt to study the haemoglobin-dithionite-peroxide reaction by this method, in spite of the evident complexity of the reaction, and the likelihood that the degradation of a considerable proportion of the haemoglobin to choleglobin and other unidentified products would preclude an unequivocal interpretation of the results. It was realised at the outset that only if the life of the transient compound was associated with diamagnetism of the reaction mixture would the results be of definite value in identifying the compound. This has not proved to be the case. Nevertheless, the results obtained are additional proof of the formation of a transient compound which slowly reverts to haemoglobin, and yield a tentative value for the molar susceptibility of the compound.

MATERIALS

Oxyhaemoglobin. Solutions of human oxyhaemoglobin were prepared from haemolysates of washed red cells, from which catalase was removed by adsorption on alumina. The solutions were dialysed against water, and finally against borate buffer solutions of pH 8.5 or 9.5. The haem content was estimated spectrophotometrically as the cyanmethaemoglobin derivative (Drabkin and Austin [8]). Details of such preparations have been given by Dalziel and O'Brien [2]. The iron content, estimated by a slight modification of the method of Lorber [9], was always about 5 % greater than the haem content.

Sodium Dithionite was a fresh, dry commercial sample stored in a sealed bottle.

Hydrogen Peroxide. A stock solution, *ca.* 1.5 *M*, was prepared by dilution of perhydrol with glass distilled water, and was analysed periodically by titration with potassium permanganate.

Buffer Solutions. To dilute the stock reagent solutions and prepare reaction mixtures of pH 10.0, Clarke and Lubs borate buffer solutions of four times the usual concentration were used, *viz.* 0.2 *M* H_3BO_3—KCl with the addition of the appropriate volume of 1 *N* NaOH. Even with this high buffer capacity, the acidic products of the reaction of H_2O_2 and $Na_2S_2O_4$ caused the pH to fall by 0.6 units during the reaction.

METHODS

Preparation and Sampling of the reaction mixture. In preparing the reaction mixture and filling the magnet tube and the optical cell, it was necessary to work as quickly as possible and to minimise exposure of the solutions to air. 5 ml portions of the reaction mixture were prepared in an Erlenmeyer flask fitted with a cork with a small cut for gas outlet. A glass tube passing through a hole in the cork was connected to a nitrogen cylinder; the reactants were introduced, and samples of the mixture withdrawn, through a second hole in the cork.

3 ml 0.715 mM (haem) HbO_2 was pipetted into the flask, and the air was displaced by a stream of nitrogen, which was maintained during the subsequent manipulations. 1 ml $Na_2S_2O_4$ solution, containing 2 g/100 ml and prepared and stored under nitrogen in a Thunberg tube, was added, followed after mixing by 0.1 ml 0.1 M H_2O_2. The contents of the flask were immediately mixed and a stopwatch started. A sample was quickly withdrawn into a Pasteur pipette, from which portions were delivered into the optical cell and the magnet tube, all previously flushed with nitrogen.

The magnet tube and optical cell were kept at the working temperature in their respective compartments until just before filling. Strict temperature control of the solutions during the preparation and sampling was not possible, but for the experiments below room temperature the reagents and pipettes *etc.* were first brought to the experimental temperature in the magnet thermostat.

Spectrophotometric Measurements. A Beckman quartz spectrophotometer model DU was used. The cell compartment was kept at constant temperature by thermostat water circulation in double-layer thermospacers. Optical depths of 0.1—0.02 cm were obtained by means of 1 cm cells into which solid quartz prisms 0.90—0.98 cm thick were inserted. The prisms carry lids which overlap the edges of the cell, and a layer of vaseline round these lids ensured an air tight seal.

The extinction of the reacting mixture at 430 mμ, the wavelength of the haemoglobin Soret maximum, was recorded at intervals until a constant value was reached. Time was measured with a stopwatch. The earliest measurement which could be made by this technique was about 2 minutes after the start of the reaction. The results are recorded as millimolar extinction coefficients, $\varepsilon_{mM\ (haem)} = \log I_0/I \times 1/cd$, where c = initial haem content of the reaction mixture in mM, d = optical depth in cm. The optical depths of the cell-prism combinations were determined by calibration with oxyhaemoglobin solutions against a 1.000 cm cell.

Magnetic Measurements. The apparatus described by Theorell and Ehrenberg [7] was used in its present condition with improved air shielding, temperature control and filling technique. Details will be published elsewhere. The instrument was calibrated with a dilute solution of nickel chloride for which the molar susceptibility was assumed to be $4\,434 \times 10^{-6}$ cgs emu at 20° C[7]. The calibration constant was 2.015×10^{-11} volume susceptibility units per scale division (μ). As the errors in the deflections were about $\pm$ 4 μ it was desirable to work with solutions containing not less than 0.4 mM haem iron so that molar susceptibilities could be determined within $\pm 200 \times 10^{-6}$ cgs emu. Preliminary spectrokinetic experiments showed that the life of the transient at this concentration would be long enough for magnetokinetic measurements if the initial reaction mixture contained 0.4 g/100 ml $Na_2S_2O_4$ and 20 mmole/l H_2O_2.

The diamagnetic correction due to the protein was determined by measurements on two samples of carboxyhaemoglobin, prepared by omitting hydrogen peroxide from the reaction mixture and saturating the Hb-$Na_2S_2O_4$ solution with carbonmonoxide. The mean value was 10 μ for a sample with 0.479 mM haem and 0.504 mM iron. This is slightly less than would be expected in comparison with previous data on myoglobin [10]. The difference can be explained by a moderate persisting paramagnetism of the non-haem iron. As the behaviour of this iron in the reaction mixture is not known, we have preferred to correct with the lower figure and base all susceptibility values as well as extinction coefficients on the haem-iron content. In that way the possible error due to the foreign iron will be minimized.

The procedure and evaluation of a magnetokinetic experiment was as follows. First the counter-balance part of the magnet tube was filled with water. A blank mixture was prepared, containing all the reactants and buffers except the oxyhaemoglobin; a buffer solution, which had been equilibrated with the oxyhaemoglobin solution by dialysis, was added in place of the latter. The peroxide was added last and a stopwatch was started. The sample part of the magnet tube was quickly filled and the tube brought in measuring position. Readings were taken alternately with 0 A and 20 A through the magnet-coil until stable readings were obtained. The time was taken at every reading. The procedure was repeated with a sample of the reaction mixture, and finally once again with the buffer-blank. The readings at 0 and 20 A for the buffer blanks, and at 0 A for the reaction mixture were plotted against time and smoothed-out curves drawn. Values at the instants when the reaction mixture was read at 20 A were interpolated on these five curves. The difference reading of the reaction mixture was $\Delta S_{\text{reaction mixture}}$

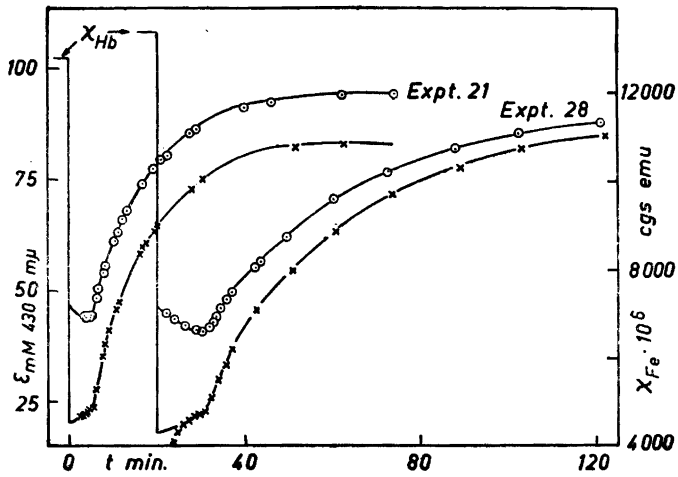

Fig. 1. The magnetic susceptibility, χ_{Fe} (×), and the extinction at 430 mμ, ε_{mM} (o), of two experiments as functions of time. Expt. 21 is made at 21.6° C and starts at 0 time, while expt. 28 is made at 13.0° C and starts at 20 min. The initial susceptibility values, χ_{Hb}, are indicated.

$= S_{20} - S_0$. This value minus the mean difference reading of the two buffer experiments gives the deflection S'. To that value we have further to add the diamagnetic correction $S_{diam} = 10 \cdot \frac{c_{haem,\ actual}}{c_{haem,\ diam}}$, where $c_{haem,\ diam}$ is the haem content in the experiment that gave the diamagnetic correction 10 μ. Thus the corrected deflection S is obtained and can be inserted in the formula

$$\chi_{Fe,\ haem} = \frac{K \cdot S}{c_{haem}}$$

where K is the calibration constant and c_{haem} is in moles per ml so that χ is obtained in cgs emu. The susceptibility values were then plotted *versus* the time.

Because of drifts in buffer readings and readings at 0 A during the first minutes of the measurements, and because of the foreign iron present, we estimate the probable errors in the χ values during that period of the reaction to be as high as $\pm$ 500 × 10^{-6} cgs emu.

RESULTS

The results of two typical experiments, one at 21.6° C (Expt. 21) and the other at 13.0° C (Expt. 28), are shown in Fig. 1. The composition of the reaction mixture in both experiments was 0.43 m*M* haem, 0.4 % $Na_2S_2O_4$ and 20 m*M* H_2O_2. The pH decreased from 9.9 to 9.3 during the overall reaction. The magnetic susceptibility, χ_{Fe}, and the extinction at 430 mμ, ε_{mM}, are plotted against the time, t. The peroxide was added to the haemoglobin-dithionite solution at $t = 0$ in Expt. 21 and at $t = 20$ minutes in Expt. 28. The initial susceptibility values $\chi_{Fe} = 12\,700$ and $13\,300 \times 10^{-6}$ cgs emu, respectively, are shown in Fig. 1, but the initial extinction level, $\varepsilon_{mM} = 145$, is omitted; the data for room temperature were obtained in control experiments, in which the peroxide was replaced by buffer solution, and the susceptibility value of Hb at 13° C was calculated by means of Curie's law.

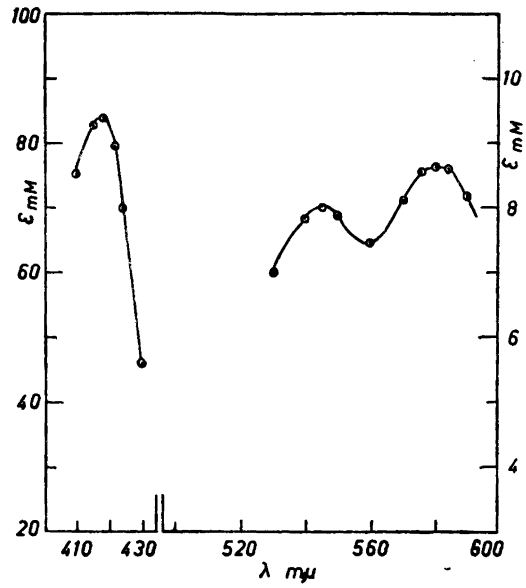

Fig. 2. The absorption spectrum of the reacting mixture at 13.0° C, in the Soret-region 4 min. and in the visible 5 min. after the start of the reaction.

It is obvious that ε_{mM} and χ_{Fe} change in a similar manner during the reaction. In both experiments there are marked decreases from the initial haemoglobin levels to the first measurements 2—3 minutes after the addition of peroxide. There is then a period during which both parameters change quite slowly, ε_{mM} decreasing and χ_{Fe} increasing, which lasts for 5 minutes at 21.6° and 11 minutes at 13.0°. Finally, both parameters increase in a roughly exponential manner at a similar rate, with a half-time of approximately nine minutes at 21.6° and twenty-one minutes at 13.0°. The stable end-values are ε_{mM} = 90 —95 and χ_{Fe} = 10 800. In Expt. 28 there is an inflexion in the χ_{Fe} curve at 5 minutes for which there is no parallel in the ε_{mM} curve. This is almost certainly an artefact, however, due to errors in the first two susceptibility measurements associated with the sample and the magnet tube regaining the experimental temperature of 13° after handling at room temperature. Some control experiments were made at 13° C with samples of known and constant susceptibility. A few of them showed steep starting branches similar to that of Expt. 28. The slow increase of χ_{Fe} from 5 to 11 minutes, however, is real and reproducible.

Both the magnetokinetic and the spectrokinetic curves indicate the occurrence of at least three successive reactions. The first stage was complete by the time the earliest measurements were made, and was accompanied by profound changes of spectral absorption and magnetic susceptibility; the haemoglobin must therefore have undergone reaction involving the iron atoms. The final reaction was the regeneration of haemoglobin, and results in partial reversal of the initial changes of both parameters. The overall reaction results in a decrease of about 35 % in ε_{mM} at 430 mμ, and at least this proportion of the

haemoglobin must therefore have been destroyed. The choleglobin formed does not account for the whole of this loss, but apart from a choleglobin maximum at 630 mμ there was no evidence for the formation of other products with significant spectral absorption (Dalziel and O'Brien [3]). The net decrease of magnetic susceptibility is about 15 %, so that the mean susceptibility of the non-haemoglobin iron in the products must be about $8\,000 \times 10^{-6}$ cgs emu. The liberation of free ionic iron by oxidative fission of haem is obviously likely to occur.

During the middle period, which lasts from the earliest measurements up to 5 minutes in Expt. 21 and 11 minutes in Expt. 28, ε_{mM} decreases in an approximately linear manner. It has been shown by Dalziel and O'Brien that during the same period the extinction at many other wavelengths decreases at a similar rate, and that consequently a distinctive spectrum persists without appreciable distortion. The presence of this spectrum under the conditions of Expt. 28 was established by measurements of the spectral absorption of fresh reaction mixtures made up from the same reagents under identical conditions. The important spectral regions were scanned back and forth in the period 2—8 minutes after the addition of the peroxide, so that the means of pairs of extinction measurement at each wavelength corresponded to the same time from the start of the reaction. The absorption spectrum of the reacting mixture 5 minutes after the start of the reaction is shown in Fig. 2 and agrees closely with the transient spectrum recorded by Dalziel and O'Brien[3].

The magnetic susceptibility measurements during the last half of the life of this transient spectrum in both the experiments (Fig. 1) show clearly that it is associated with a slowly increasing susceptibility value of about 4 500. The coincidence, in both experiments, of an inflexion in the χ_{Fe} curve with the minimum in the ε_{mM} curve which marks the end of the life of the transient spectrum, and the onset of haemoglobin regeneration, is striking.

DISCUSSION

The changes of magnetic susceptibility which follow the addition of hydrogen peroxide to a haemoglobin solution containing excess dithionite confirm the conclusions drawn by Dalziel and O'Brien [3] from spectrokinetic studies regarding the general course of the reaction. The haemoglobin is temporarily converted, in a rapid reaction involving the iron atoms, into at least one unstable derivative which possesses distinctive spectral absorption and rather low magnetic susceptibility, and from which a high proportion of the haemoglobin is subsequently regenerated. As regards the detailed course of the reaction, the magnetic data taken alone do not give any new information.

The formation of a distinctive transient spectrum was demonstrated again under the conditions of the present experiments a few minutes after the start of the reaction. When the measured extinction coefficients (Fig. 2) are corrected for the decrease of intensity which occurs during the life of the spectrum by extrapolation to zero time values of $\varepsilon_{mM} = 88$, 8.6 and 9.2 at 418, 545, and 580 mμ are obtained. The transient spectrum is therefore in excellent quantitative agreement with that recorded by Dalziel and O'Brien [3] a few seconds after the start of the reaction.

It is possible that this transient spectrum characterises a single derivative of haemoglobin [3], and the principle objective of the present experiments was to see whether it was associated with a reasonably constant and reproducible magnetic susceptibility value. The measurements have shown that at least during the last half of the life of the transient spectrum the magnetic susceptibility of the reaction mixture increases slowly. This observation can be explained by the slow degradation of the transient compound to colourless products of high susceptibility which is suggested by a concomitant slow decrease of intensity of the spectrum, and the fact, already mentioned, that some of the degradation products formed in the overall reaction have relatively high susceptibility. If this slow increase of susceptibility persists throughout the life of the transient compound, the best estimate of the susceptibility of the compound will be obtained by extrapolation to zero time. In the two experiments described here, values of 4 500 and 4 300 $\times 10^{-6}$ cgs emu are so obtained; other experiments gave values of from 4 300 to 5 000 $\times 10^{-6}$ cgs emu. Since the errors of the measurements are rather large, and nothing is known about the validity of Curie's law in this instance, a mean value was taken, regardless of temperature differences between the experiments. Thus, a value of 4 500 $\times 10^{-6}$ cgs emu was obtained for the molar susceptibility of the transient compound, on the assumption that all the haemoglobin is converted into a single derivative by the initial rapid reaction with hydrogen peroxide. Susceptibility values of this order are common to hydrogen peroxide and methyl hydrogen peroxide compounds of type II and III of peroxidase, catalase and metmyoglobin [6]: HRP—MeOOH—II, $\chi_M = 5\,040 \times 10^{-6}$; Cat—MeOOH—II, 3 400; metMb—MeOOH—III, 3 000; metMb—H_2O_2—III, 3 500. These data have been interpreted as due to two unpaired electrons and the proposed structure is a resonance between a ferric-radical form and a ferryl form [11]. Judged from the susceptibility value the same structure might be proposed in the case of the transient.

The position of the absorption bands of the transient in the visible, 545 and 582 mμ, would also suggest a compound of type III. The relative height of the two absorption bands, $\varepsilon_{445} < \varepsilon_{582}$, is, however, contrary to what would be expected for such a compound. On the other hand, the transient spectrum is also consistent with a ferrohaem derivative. Theoretically the iron in such a compound have two unpaired electrons and square dsp^2 bonds, which would be compatible with the susceptibility reported here. Among the ferrohaem derivatives, however, no such compound has yet been demonstrated, and although there is no experimental evidence against this interpretation, it is not a likely one.

Thus the present investigation supports the view that the transient spectrum is predominantly due to a peroxide derivative of haemoglobin similar to those already recognised for peroxidase, catalase, and myoglobin, but the question whether it is due to metHb—H_2O_2—III mixed with some other derivative with an absorption maximum at about 580 mμ, or to a new compound, remains unanswered. Direct comparison with the susceptibility values of metHb-peroxide-III compounds can not be made at the moment, as these data are still lacking. We hope to determine them in the near future.

With the present technique, magnetic measurements could be made only during the last half of the life of the transient spectrum, partly because of the time taken to prepare the sample and fill the magnet tube *etc.*, and partly because inadequate temperature control caused inaccuracies in the first few measurements at low temperature, when the life of the spectrum is longest. It should be possible to improve the technique in both these respects. The first measurement could probably be made 1 ½ minutes after the start of the reaction. At a temperature of 2° C we found the life of the transient to be 21 minutes, but an attempt to make magnetic measurements at this low temperature failed because of condensation on the optical system of the magnet, which must also be cooled down. If magnetic measurements could be made under these conditions it would be possible to test the association of the transient spectrum with a definite susceptibility value much more conclusively.

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Studies on Polynuclear Complexes

I. Formation of Polynuclear Hydroxo Complexes in Aged Solutions of Diaquobis(ethylenediamine)cobalt(III) Ions and Kinetics of Decomposition of μ-Dihydroxotetrakis(ethylenediamine)cobalt(III) Ion

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The primary hydrolysis products of the Co $en_2(H_2O)_2^{+++}$ ion are the monomeric aquohydroxo and dihydroxo ions. During a slow ageing process polymeric hydrolysis products are formed, but no equilibrium state seems to be obtained even after ageing for years. The rate of the slow hydrolysis is lower in paraffined glass than in ordinary glass vessels. It is shown that μ-hydroxo- and μ-dihydroxo dicobalt ions are not among the hydrolysis products. The tetrakis(ethylenediamine)-μ-dihydroxo ion is found to be decomposed to monomers which react slowly to form new polynuclear ions. The decomposition of this μ-dihydroxo salt is studied in detail, and a mechanism for the decomposition in acidic solution is proposed.

In a previously published paper [1] the system of acid-base and *cis-trans* equilibria of the diaquobis(ethylenediamine)cobalt(III) ions was examined. During our work with this system we noticed that solutions in the buffer range were slowly transformed with an increasing hydrogen ion concentration indicating that polynuclear hydrolysis products were being formed. At the same time changes in the absorption spectrum were observed. For a closer investigation of these phenomena we first carried out a redetermination of the first gross acidic dissociation constant ($K_{s,1}$(gr.)) of *cis-trans* equilibrium mixtures of the diaquobis(ethylenediamine)cobalt(III) ions. Using the same symbols as in Ref.[1], the average number of hydrogen ions produced by the diaquo ions (= number of hydroxyl ions bound per cobalt atom) is given by

$$\bar{\nu} = \frac{C_{NaOH} + [H^+] - [OH^-]}{C_{Co}}$$

if $C_{NaOH} \lesssim 2\ C_{Co}$ and the first gross dissociation constant by

$$-\log K_{s,1}(\text{gr.}) = -\log [H^+] + \log \frac{1-\bar{\nu}}{\bar{\nu}} + \log (1 + \Delta)$$

when $\Delta = \dfrac{(2-\bar{\nu})\ K_{s,2}(\text{gr.})}{(1-\bar{\nu})\ [H^+]}$.

Table 1. The first gross acidic dissociation constant of the diaquobis (ethylenediamine) cobalt (III) ions in 1 M sodium nitrate at 25° C.

Solutions are made from cis[Co en_2aq_2]$(NO_3)_3$, and all concentrations are given in moles per liter.

$C_{NaNO_3} = 1.00\ M$, $-\log\ [H^+]_{st.} = 2.297$, $-\log\ K_{s,2}(gr.) = 8.10$.

C_{Co}	C_{NaOH}	$\bar{\nu}$	$E_{st.}-E$	$-\log [H^+]$	$\log \frac{1-\bar{\nu}}{\bar{\nu}}$	$\log (1+\Delta)$	$-\log K_{s,1}$ (gr.)
0.01074	0.002708	0.2524	179.6	5.336	0.472	0.002	5.810
0.009936	0.003256	0.3277	189.4	5.502	0.312	0.003	5.817
0.009998	0.004570	0.456	202.9	5.729	0.077	0.005	5.811
0.01013	0.005064	0.500	207.4	5.806	0.000	0.006	5.812
0.01005	0.006089	0.6011	217.4	5.975	−0.178	0.011	5.808
0.01016	0.006609	0.650	222.6	6.063	−0.269	0.015	5.811
0.09950	0.007460	0.7495	234.0	6.257	−0.476	0.030	5.811

Mean 5.811 ± 0.003

The measurements are recorded in Table 1. The value of $K_{s,2}$(gr.) was taken from the previous paper to be $10^{-8.10}$. The mean value found $K_{s,1}$(gr.)$= 10^{-5.811 \pm 0.003}$ is in very good agreement with the previously found value $10^{-5.80}$. The constancy of $K_{s,1}$(gr.) shows that only mononuclear hydrolysis products

Table 2. Changes in time of pH and extinction coefficients of cis-trans equilibrium mixtures made from cis-diaquo nitrate, and of solutions of "diol" nitrate

$C_{NaNO_3} = 1.00\ M$, 25° C

No	Salt	C_{Co}	$\bar{\nu}$	$-\log [H^+]$	Age in days	Vessel	540	520	500	440	420	410	380	360 mμ
1	diaquo	0.01074	0.252	5.336	1	paraff.	48.2	67.4	78.9	30.2	21.1	23.1	54.1	69.4
				5.283	95	»	48.8	67.6	80.6	30.6	20.7	22.6	54.7	71.0
				4.867	506	»	51.1	72.9	85.9	29.4	18.1	19.7	54.8	74.8
2	diaquo	0.01013	0,50	5.806	1	paraff.	50.9	66.6	74.5	31.0	26.6	30.9	59.8	69.7
				5.047	481	»	56.3	79.0	89.1	28.6	18.9	21.8	69.2	80.0
				6.673	1	Norite *	47.3	61.2	67.9	35.8	36.5	43.2	88.4	138.2
3	diol	0.00989	0.49	2.300	$t = 0$	glass	145.9	151.2	124.2	35.7	56.2	75.5	122.9	132.2
				5.839	13	»	52.4	68.3	75.6	31.2	27.4	32.0	61.4	71.4
				5.107	423	»	58.8	81.9	91.9	28.9	19.4	22.8	61.5	82.6
4	diaquo	0.00995	0.75	6.257	1	paraff.	54.7	67.8	71.4	31.9	31.9	38.5	66.1	71.7
				6.187	482	»	57.5	71.3	75.5	32.8	32.8	39.8	71.0	81.2
5	diaquo	0.00855	1.00	6.902	1	glass	58.9	70.1	70.5	33.4	37.2	45.9	73.6	76.5
				5.577	422	»	71.5	96.2	100.7	28.8	23.8	30.1	78.5	109.2
6.	diol	0.00974	1.00		$t = 0$	glass	same spectrum as solution 3 at zero time							
				6.991	12		60.6	71.0	70.2	32.6	37.0	46.1	74.6	76.9
				5.747	422		70.2	93.7	97.8	28.1	24.0	30.4	76.0	99.8

* 50 ml of solution 2 were shaken with 0.035 g Norite charcoal for 20 hours in a thermostat at 25° C. The spectrum was taken after filtration from the charcoal.

are formed instantaneously. In the course of some weeks or months, however, the solutions show measurable changes. The rate of change is dependent upon the surface of the reaction vessel. Solutions stored in paraffined bottles change rather slowly and irregularly, one of them did not change at all in the course of 400 days. Solutions which were kept in ordinary glass bottles changed more rapidly. Charcoal has a striking effect. Evidently it catalyzes a splitting off of ethylenediamine as its effect is to decrease $[H^+]$. In Table 2 a selection of the experimental data is presented. It is seen from experiment No. 2 that both the spectral changes and the changes in $[H^+]$ in the solution treated with charcoal are quite different from those of the other experiments.

An explanation of the observed phenomena was first sought in the process:

$$2\ \mathrm{Co\,en_2(H_2O)_2^{3+}} \rightleftharpoons \mathrm{en_2\,Co\langle^{OH}_{OH}\rangle Co\,en_2^{4+}} + 2\ \mathrm{H^+} \qquad (1)$$

and for testing this hypothesis the well-known tetrakis(ethylenediamine)-μ-dihydroxodicobalt(III) nitrate, Werner's "Diolsalt" was prepared. For shortness it is referred to as "diol" in the following.

For solutions of this compound $\bar{\nu}$ is defined as the number of hydroxyl ions bound per cobalt atom after monomerization:

$$\bar{\nu} = \frac{C_{Co} - C_{HNO_3} + C_{NaOH} + [H^+] - [OH^-]}{C_{Co}}$$

if $|C_{HNO_3} - C_{NaOH}| \lesssim C_{Co}$.

Tables 2, 3 and 5 show that in solution the "diol" compound is decomposed to the monomeric system for all values of $\bar{\nu}$. Solutions of "diol" salt obtain in the course of some days an absorption spectrum which is identical with that of freshly prepared solutions of diaquosalt at the same $[H^+]$. Later on, the "diol" solutions in the buffer range change in the same way as do the solutions which are made up from the diaquosalt. These experiments definitely rule out the possibility of explaining the ageing process by equation (1).

Another simple explanation might be found in the reaction:

$$\mathrm{Co\,en_2(H_2O)OH^{2+} + Co\,en_2(H_2O)_2^{3+} \rightleftharpoons H_2O\,Co\,en_2\text{-}OH\text{-}Co\,en_2H_2O^{5+} + H_2O} \qquad (2)$$

According to this mechanism the change in absorption spectrum should be greatest in solutions of $\bar{\nu} = 0.5$ and the hydrogen ion concentration should stay constant at this $\bar{\nu}$. Furthermore, it should decrease when $\bar{\nu} < 0.5$ and increase when $\bar{\nu} > 0.5$. It can be seen from Table 2 that this possibility must be ruled out too *.

As the results were not very reproducible and as a final equilibrium state was apparently never reached it seems meaningless to apply any of the elaborate theories of continuous polymerization *e. g.* those of Sillén [2]. The *trans* ions have possibilities of forming chains while the *cis* ions might form large, strain-free rings but it seems impossible to give any satisfactory solution of the problem.

* In Abstracts of Papers of the XIIIth International Congress of Pure and Applied Chemistry, Stockholm 1953, the reaction (2) was suggested by us prematurely on the basis of preliminary experiments.

From the data in Table 2, however, one would be inclined to believe that the equilibrium cannot be displaced very far towards the polymer side *. This is supported by the fact that the aged solutions when acidified, develop the spectrum of the monomeric diaquo ions within a few minutes.

It is seen from Tables 2, 3 and 4 that the absorption spectrum of the "diol" is different in alkaline and in acid solution. Preliminary measurements showed that it is too weak an acid to have its dissociation constant determined accurately by glass electrode measurements but $K_{s,1}$ is probably of the order of magnitude 10^{-10}.

KINETICS OF THE DECOMPOSITION OF THE "DIOL" COMPLEX

The absorption spectra of the "diol" complex in alkaline solution and those of the *cis*- and *trans*-dihydroxobis(ethylenediamine)cobalt(III) ions do not differ very much, but we have tried to analyse the spectra obtained during the decomposition, assuming that the "diol" and the *cis*- and *trans*-dihydroxo ions were the only absorbing species present in the reacting mixture. A representative run is shown in Table 3. Using the observed extinction coefficients at the given wavelengths the concentration of each of the three species can be determined as the extinction coefficients of the pure species are known [1], but the accuracy is rather low. The result of such a computation is given in Table 4, where the distribution of the cobalt among the three species is estimated applying the method of least squares. The results seem to indicate that the *cis*-ion is formed first, and then slowly isomerizes (see Ref.[1]) until equilibrium is obtained. This would be expected from the supposed configuration for the "diol" compound. A detailed kinetic investigation was not possible.

As mentioned above, the equilibrium in acidic solution is strongly displaced in favour of the *cis*-diaquo ions, which have an absorption spectrum differing considerably from that of the "diol" compound in the region 520—

Table 3. Decomposition of "diol" in alkaline solution.

$C_{Co} = 0.00510\ M$, $C_{NaOH} = 0.0132\ M$, $C_{NaNO_3} = 1.00\ M$, 25° C.

$\bar{\nu} = 2$	Time in min.	560	540	520	500	380	370	360 mμ
ε_{diol}	$t=0$ (extrapol.)	86.8	103.0	101.3	90.9	141.8	142.8	129.9
	128	69.5	88.5	96.0	85.7	116.8	120.7	111.0
	194	64.3	83.0	91.0	82.1	108.2	112.0	103.0
	244	62.6	81.0	89.5	81.0	104.8	108.0	100.2
	339	61.7	79.4	87.1	78.8	99.0	102.1	94.4
	$\sim 10^4$ (7 days)	46.6	63.6	71.5	64.8	77.0	78.9	73.1
$\varepsilon_{eq.}$		46.7	63.8	71.3	64.6	75.5	77.3	71.5
ε_{cis}		55.4	82.1	93.0	85.5	97.5	103.6	95.5
$\varepsilon_{tr.}$		38.6	49.3	53.0	48.1	54.9	53.9	50.5

* Because of the low solubility of the *trans* aquo hydroxo nitrate it was impossible to increase the cobalt concentration significantly.

Table 4. Estimated distribution of the cobalt on the species: Diol ($a_{diol.}$), cis-dihydroxo (a_{cis}), trans-dihydroxo ($a_{tr.}$) at various times in the experiment given in Table 3.

t (min.)	a_{diol}	a_{cis}	a_{trans}
0	1	0	0
128	0.482	0.470	0.048
194	0.371	0.491	0.138
244	0.321	0.513	0.166
339	0.264	0.509	0.227
$>10^4$	0	0.444	0.556

560 mμ, and there is an isobiestic point at 443 mμ. The extinction coefficient of "diol" $\varepsilon = 33.8$ at this wavelength is unchanged during the decomposition. Assuming that the "diol" and the *cis*-diaquo ion are the only species present in the reacting mixture, a degree of reaction $\alpha = \frac{\varepsilon_{diol} - \varepsilon}{\varepsilon_{diol} - \varepsilon_{cis}}$ can be calculated from the spectra (see Table 5). At a given time the degrees of reaction calculated from different wavelengths agree satisfactorily. Thus intermediate products can only exist in very small quantities.

The experiments were carried out in glass vessels before the different rates of polymerization in glass and in paraffin were found. Later on a single experiment showed that this reaction is also catalyzed by charcoal. It did not, however, seem worthwhile to repeat the measurements in paraffined bottles, as the experiments were fairly reproducible in glass apparatus. 1 *M* sodium nitrate was used as a constant salt medium in all cases.

The rate of decomposition in acidic solution is described by the following expression:

$$-\frac{dx}{dt} = k'(C^0_{Co} - x)\,(C^0_{H+} + k'' - x)$$

Table 5. Decomposition of "diol" in acidic solution.

$C^0_{Co} = 0.001792$ *M*, $C^0_{HNO_3} = 0.004065$ *M*, $C_{NaNO_3} = 1.00$, 25° C.

$\bar{\nu} = 0$	Time in min.	560	540	520 mμ	Average
ε_{diol}	$t = 0$ (extrapol.)	114.5	145.9	151.2	
	59.5	90.1	117.2	126.7	
$a_{59.5}$		0.274	0.278	0.280	0.278
	98.0	75.5	99.9	112.7	
$a_{98.0}$		0.437	0.445	0.440	0.441
	127.8	67.3	90.1	104.4	
$a_{127.8}$		0.528	0.539	0.535	0.534
	169.8	57.8	79.6	95.2	
$a_{169.8}$		0.635	0.641	0.640	0.639
	$\sim 10^4$ (7 days)	25.9	43.3	64.9	
ε_{cis}		25.5	42.8	64.6	

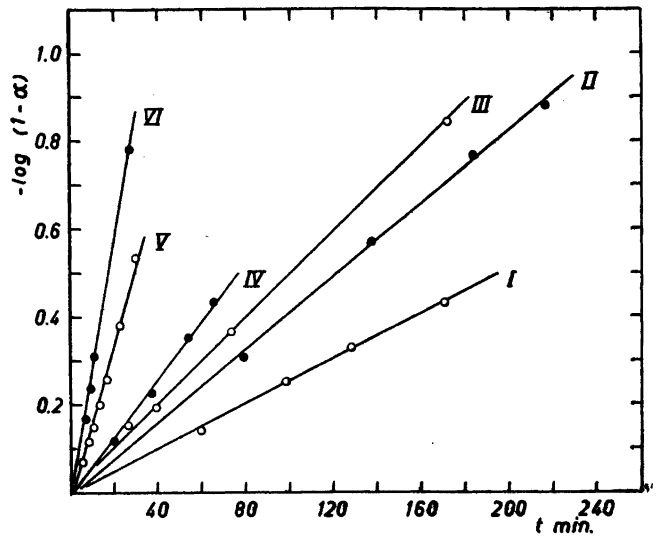

Fig. 1. $-\log(1-a)$ *vs* t *in minutes. The slopes of the lines give preliminary values of* $k_0 = k'k'' + k'(C^0_{H+} - C^0_{Co})$. *The compositions of the solutions* (C^0_{H+}, C^0_{Co}) *are the following:* I *0.00407 M, 0.00179 M,* II *0.00813 M, 0.00188 M,* III *0.0122 M, 0.00200 M,* IV *0.0162 M, 0.00197 M,* V *0.0500 M, 0.00203 M,* VI *0.1004 M, 0.00150 M.* C_{NaNO_3} = *1.00 M in all cases.*

where C^0_{H+} is the initial concentration of hydrogen ions, C^0_{Co}, moles of cobalt per liter, is the initial concentration of "diol" salt and x the concentration of cobalt transformed.

The integrated equation is

$$-\log(1-a) + \log\frac{C^0_{H+} + k'' - x}{C^0_{H+} + k''} = k'(C^0_{H+} - C^0_{Co} + k'')t$$

where $a = \frac{x}{C^0_{Co}}$ is the degree of reaction calculated for "diol" in the experiment given in Table 5. The constants k' and k'' are obtained in the following way: First for each run $-\log(1-a)$ is plotted against t and preliminary values of $k_0 = k'k'' + k'(C^0_{H+} - C^0_{Co})$ are obtained. This plot is given in Fig. 1. The k_0's are plotted against $C^0_{H+} - C^0_{Co}$ and approximate values of k' and k'' are found. The k'' values are now inserted in the log $\frac{C^0_{H+} + k'' - x}{C^0_{H+} + k''}$ term, and the left hand side of the integrated equation is plotted against t and new values of k_0 are found, which again give better values of k' and k''. Already the second iteration gave no change in the constants and the following values were found using decadic logarithms: $k' = 0.030 \pm 0.002$ liter min.$^{-1}$ equival.$^{-1}$. $k'' = 0.0060 \pm 0.0015$ equival. liter^{-1}.

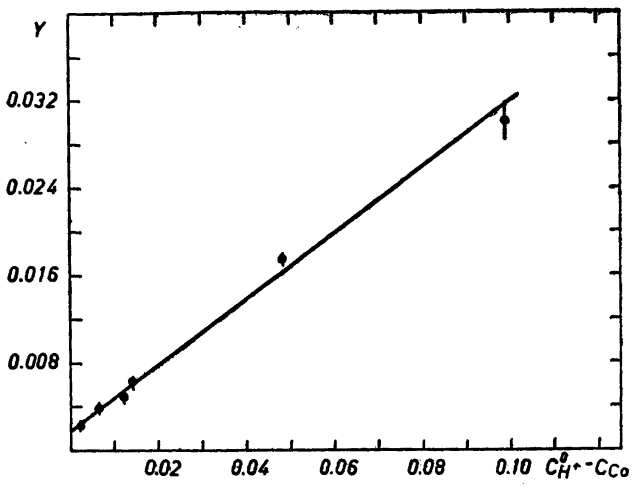

Fig. 2. $Y = -\frac{1}{t}\log(1-a) + \frac{1}{t}\log\frac{C^0_{H+} + k'' - x}{C^0_{H+} + k''}$ *where* $k'' = 0.0060$ *equival. liter*$^{-1}$ *vs* $C^0_{H+} - C^0_{Co}$. *The slope of the line gives the value of* k' *and the intercept on the y-axis gives* $k'k''$.

In Fig. 2 the experimental data are plotted using these two constants.

The rate expression found can be very simply interpreted as a combination of a "spontaneous" decomposition of the "diol" combined with a reaction with hydrogen ions:

$$en_2Co\begin{matrix}OH\\OH\end{matrix}Co\ en_2^{4+} + H_2O \xrightarrow{\text{slow}} 2\ Co\ en_2(H_2O)OH^{2+}$$

$$en_2Co\begin{matrix}OH\\OH\end{matrix}Co\ en_2^{4+} + H_3O^+ \xrightarrow{\text{slow}} Co\ en_2(H_2O)_2^{3+} + Co\ en_2(H_2O)OH^{2+}$$

$$Co\ en_2(H_2O)OH^{2+} + H_3O^+ \xrightarrow{\text{fast}} Co\ en_2(H_2O)_2^{3+} + H_2O$$

It might, however, be more reasonable to assume that an intermediate containing only one hydroxyl bridge might be present in small concentrations: *e. g.* the ion $[en_2Co(H_2O)—OH—(H_2O)\ Co\ en_2]^{5+}$. Assuming that a steady state is rapidly obtained with constant concentration of this ion, the above mentioned rate equation is again obtained.

If this interpretation be true the rate of the "spontaneous" reaction in glass vessels at 25° and in 1 *M* $NaNO_3$ is given by $k'k'' = 0.00018$ min.$^{-1}$ and that of the reaction with hydrogen ions by $k' = 0.030$ liter min.$^{-1}$ equival.$^{-1}$.

EXPERIMENTAL

The *cis*-diaquobis(ethylenediamine)cobalt(III) nitrate was prepared and analysed as in Ref.[1] (Found: Co 13.92; N 23.11. Calc. for Co $en_2(H_2O)_2(NO_3)_3$, H_2O: Co 14.06, N 23.39.)

The tetrakis(ethylenediamine)-μ-dihydroxodicobalt(III) nitrate was prepared according to Werner [3] from *cis*-aquohydroxobis(ethylenediamine)cobalt(III) dithionate which was heated in an electric furnace at 110° C until constant weight was obtained.

The "diol" dithionate was obtained in this way but simultaneously another compound was formed which was insoluble both in acids and in bases. It is probably a high polymer although crystalline as shown by an X-ray diffraction pattern.

The "diol" nitrate was obtained from the dithionate *via* the bromide as described by Werner [3]. Contrary to Werner it was found to contain water. (Found: Co 18.12, C 14.52, H 5.48, N 25.71. Calc. for $[en_2Co\langle^{OH}_{OH}\rangle Co\ en_2](NO_3)_4, 1/2\ H_2O$: Co 18.16, C 14.80, H 5.43, N 25.89 *.)

The glass electrodes were of the MacInnes and Dole type [4]. They were calibrated and found to have the theoretical response at 25° C. The hydrogen ion concentration was determined in all cases by standardizing against a standard acid solution of the composition $C_{HNO_3} = 0.005050$, $C_{NaNO_3} = 1.000$. The measurements were performed in a paraffin oil thermostat which controlled the temperature within ± 0.02° C. The valve potentiometer was a "Radiometer" Model PHM 3. The liquid junctions were established with cylindrical symmetry as recommended by Guggenheim [5] using saturated KCl as a bridge solution. The whole set up was essentially similar to that used by Pedersen [6].

In the kinetic experiments the reaction mixtures were kept in a water thermostat with a temperature control of ± 0.01° C.

The absorption spectra were measured on a Beckman DU spectrophotometer, the wavelength scale of which had been calibrated by aid of a mercury lamp. The cell compartment and the phototube were thermostated by aid of thermospacers. The molar extinction coefficient

$$\varepsilon = \frac{\log_{10} I_0/I}{C_{Co} \cdot d}$$

(C_{Co} is the molar concentration of cobalt and d the thickness of the absorbing layer in cm) is given in all cases.

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Ozonization of Some Benzodifurans

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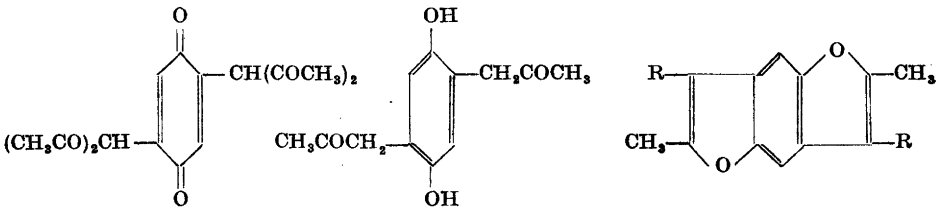
Three derivatives of benzo [1.2-b, 4.5-b'] difuran have been ozonized. Ozone was rapidly added to the furan double bonds. The products of decomposition are discussed. In one instance remarkably stable and well-crystallizing ozonides were obtained.

The base-catalyzed reaction of acetylacetone with *p*-benzoquinone led, as found by one of the present authors [1,2], to the new compound $\omega, \omega, \omega', \omega'$-tetraacetyl-*p*-xyloquinone (I).



I II III

On catalytic hydrogenation in methanol this substance gave, with simultaneous loss of two acetyl groups, ω, ω'-diacetyl-*p*-xylohydroquinone (II) which in turn by treatment with acetyl chloride would split off two molecules of water and thereby effect a ring closure to 2,6-dimethylbenzo [1.2-b, 4.5-b']-difuran (III, R = H). Another route to III (R = H) was found in condensing ethyl acetoacetate and *p*-benzoquinone to ethyl 2,6-dimethylbenzo [1.2-b,-4.5-b']-difuran-3,7-dicarboxylate (III, R = $COOC_2H_5$) as described by v. Pechmann [3] and Ikuta [4]. Hydrolysis of this ester and decarboxylation of the corresponding potassium salt furnished a substance identical with III (R = H).

Most of our arguments in favor of the structure III (R = H) were thus based upon an acceptance of the correctness of the structural formulae put forward by Ikuta [4]. However sound this foundation may be it was felt that some independent proof ought to be furnished.

Several modes of degradation were contemplated and that of ozonolysis chosen as the most promising. v. Wacek, Eppinger and v. Bézard [5] have carried out ozonization of benzofuran and some homologues with the substituents in the furan ring. Their experiments indicate a facile reaction with the furan double bond without any noticeable attack on the benzene nucleus. Further the yield of the expected aldehydes seems to be reasonably good and not too susceptible to the method of decomposing the ozonide.

III (R = H) was ozonized in chloroform or glacial acetic acid at room temperature with 3 to 4 % ozone. Ozone was rapidly absorbed during the first half of the reaction but escaped thereafter in increasing amounts. This is of course in agreement with the well-established fact that in a conjugated system (the two furan double bonds are conjugated through the benzene nucleus) where no aromatic bonds are attacked by ozone, addition occurs much more rapidly to the first double bond than to subsequent ones.

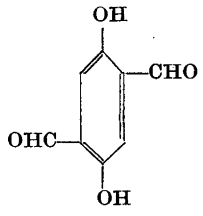
When the calculated amount of ozone had been passed into the solution decomposition was effected in two ways:

A The chloroform solutions were concentrated *in vacuo* until a syrupy ozonide remained. This was decomposed with hot water.

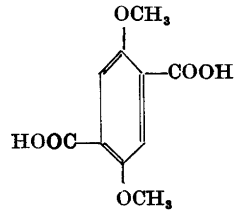
B Zinc dust was added to the glacial acetic acid solutions and the mixtures heated on a water-bath.

In both instances hydroquinone-2,5-dialdehyde (or 2,5-dihydroxyterephthalaldehyde) (IV) was obtained in 15 to 20 % yield. Other decomposition products (acids and polyvalent phenols) evaded isolation and identification.

The dialdehyde IV could be acetylated to give a diacetylderivative and reacted with *p*-nitrophenylhydrazine to give a bis(*p*-nitrophenylhydrazone). With methyl sulphate in alkaline solution it was converted into 2,5-dimethoxyterephthalaldehyde which could be oxidized by permanganate to 2,5-dimethoxyterephthalic acid (V).



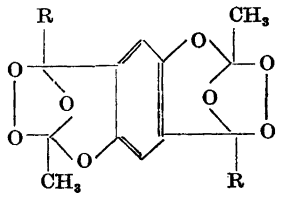
IV



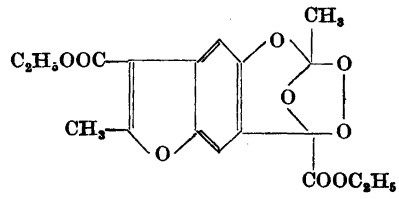
V

We considered it of interest also to try the ozonization reaction on two other available benzodifurans, *viz.* the beforementioned ethyl 2,6-dimethylbenzo-[1.2-b, 4.5-b']difuran-3,7-dicarboxylate (III, R = $COOC_2H_5$) and 2,6-dimethyl-3,7-diacetylbenzo-[1.2-b,4.5-b']difuran (III, R = $COCH_3$). III (R= $COOC_2H_5$) was ozonized in ethyl acetate and the solution concentrated *in vacuo*. The resulting syrup was interspersed with white crystals. On treatment with a small amount of ethanol these were left undissolved while the syrup went into solution. When the ethanolic solution was concentrated a second and smaller crop of the white crystals separated. The filtrate was slowly con-

centrated further when beautiful yellow crystals were formed. The mother liquor was then a reddish syrup from which no more crystalline material could be obtained. Most likely the two crystalline substances were ozonides as they burned in a flame with the typical vigour and contained active oxygen. Analysis gave the composition $C_{18}H_{18}O_{12}$ with two active oxygens for the white compound and $C_{18}H_{18}O_9$ with one active oxygen for the yellow compound. The former must have the structure VI (R = $COOC_2H_5$) and the latter the structure



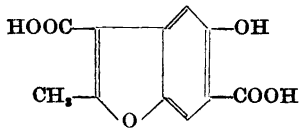
VI



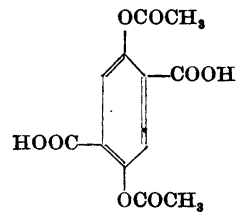
VII

VII representing a diozonide and monozonide, respectively. Determination of the molecular weights showed both ozonides to be monomeric. VI (R = $COOC_2H_5$) and VII could be recrystallized from hot alcohol or from glacial acetic acid and have now been kept for a long time at room temperature without noticeable decomposition. They are consequently of a stability rarely encountered in true ozonides of non-nitrogenous heterocycles [6].

Catalytically activated hydrogen was readily absorbed by the ozonides but only dark tarry syrups resulted from the reaction. Boiled with aqueous sodium hydroxide, VI (R = $COOC_2H_5$) was hydrolyzed to 2,5-dihydroxyterephthalic acid which was identified as the dimethyl ether (V). Under the same conditions VII yielded a dibasic acid $C_{11}H_8O_6$ which, when heated with concentrated sulphuric acid, gave the violet colour typical for benzofurans. The structure VIII was assigned to this substance whose dimethyl ester was



VIII



IX

prepared by the action of diazomethane.

2,6-Dimethyl-3,7-diacetylbenzo[1.2-b, 4.5-b]difuran (III, R = $COCH_3$) was also ozonized in ethyl acetate. After evaporation of the solvent a clear yellow syrup remained. It was dissolved in a few ml of ethanol and the solution was concentrated by slow evaporation at room temperature. In the course of several days a light yellow substance separated which showed all signs of being an ozonide. It was, however, much less stable than VI (R = $COOC_2H_5$),

being decomposed by hot ethanol for instance. No accurate analysis could be obtained but its content of active oxygen indicated that it must be the diozonide VI (R = $COCH_3$). Aqueous sodium carbonate hydrolyzed it to 2,5-dihydroxyterephthalic acid. The syrupy mother liquor from VI (R = $COCH_3$) was placed in a refrigerator when a new white substance separated. It was obviously an acid and when hydrolyzed in aqueous sodium carbonate it yielded 2,5-dihydroxyterephthalic acid. With diazomethane it gave 2,5-diacetoxyterephthalic acid dimethyl ester and must have the structure IX which is one of the decomposition products expected from the diozonide VI (R = $COCH_3$). The noncrystallizing syrup left when IX was removed furnished on hydrolysis a good yield of 2,5-dihydroxyterephthalic acid.

EXPERIMENTAL

(Melting points not corrected)

Ozonization of III (R = H)

III (R = H) (3 g) was dissolved in the appropriate solvent (chloroform or glacial acetic acid) (150 ml) and a current of oxygen (*ca.* 30 l h^{-1}) containing 4.5 % (wt.) ozone was passed through the solution until the calculated amount plus 40 % was absorbed. Unabsorbed ozone in the exit gas was measured from time to time by reaction with aqueous potassium iodide. The 40 % excess is supposed approximately to make up for the amount of ozone decomposed by the solvent before reacting with the benzodifuran. Reaction temperature was 0° with chloroform as a solvent and room temperature with glacial acetic acid.

Decomposition with hot water: The chloroform was removed *in vacuo* at 20°. The residue was a viscous yellow syrup, burning explosively and containing active oxygen. Due to insuperable difficulties in purification of the ozonide the content of active oxygen was not determined. After adding water the mixture was heated on a water-bath for 30 to 45 minutes. The resulting reddish-brown solution was allowed to cool when a brown substance separated. This heterogenous mixture was exhaustively extracted with ether. The combined extracts were shaken with a saturated solution of sodium hydrogen carbonate until the evolution of carbon dioxide had ceased. On acidifying this solution yielded a yellowish-brown substance which could not be identified. By now the ethereal solution had a clean yellow colour and after drying it was concentrated to a small volume when a crystalline yellow substance separated (IV).

Reductive decomposition: To the glacial acetic acid solution zinc dust was added and the mixture heated on a water-bath for 30 to 45 minutes with occasional stirring. The reddish-brown solution was filtered and evaporated to dryness *in vacuo*. Extraction with ether gave a solution which was treated as in the above experiment. The yield of the yellow substance (IV) was roughly the same (0.4—0.5 g) in both cases.

IV dissolved in alkali with an intense violet colour, gave a blue-violet colour with ferric chloride and reduced Tollen's reagent in the cold. It was not very volatile in steam. Recrystallized from benzene it decomposed at 240—245°. (Found: C 58.19; H 3.71. Calc. for $C_8H_6O_4$: C 57.83; H 3.64.

Acetylation: IV (79 mg) was refluxed with acetyl chloride (20 ml) until the solution was colourless (*ca.* 3 hours) and then poured cautiously into ice-water containing some sodium hydrogen carbonate and stirred briskly. The precipitated white substance (110 mg) was filtered off immediately and recrystallized from ligroin. White needles with m. p. 122—123°. In water or ethanol the substance seemed to decompose rapidly giving a yellow colour. (Found: C 57.48; H 4.11. Calc. for $C_{12}H_{10}O_6$: C 57.60; H 4.03.)

p-Nitrophenylhydrazone: To a hot solution of IV (0.5 g) in ethanol (35 ml) was added *p*-nitrophenylhydrazine (1.5 g) in ethanol (100 ml) and glacial acetic acid (5 ml). The mixture was heated on a water-bath for 30 minutes and the separated substance (0.95 g) filtered off. Recrystallized once from nitrobenzene and twice from glacial acetic acid and

dried *in vacuo* at 100° over KOH for some 10 hours. Decomposed above 310° without melting. (Found: C 54.99; H 3.77; N 18.3. Calc. for $C_{20}H_{16}N_6O_6$: C 55.04; H 3.70; N 19.26.)

Methylation: IV (1 g) was dissolved in aqueous sodium hydroxide (10.5 ml, 1 *N*). To this solution was added methyl sulphate (1.1 ml) and the mixture shaken well. Methyl sulphate (1 ml) and sodium hydroxide (10 ml, 1 *N*) were added four times with shaking between each addition. Unreacted methyl sulphate was then decomposed with excess sodium hydroxide at room temperature. The separated substance (0.35 g) was washed repeatedly with water and recrystallized from ethanol. M. p. 205–206° dec. (Found: C 61.70; H 5.28; OCH_3 31.00. Calc. for $C_8H_4O_2(CH_3O)_2$: C 61.85; H 5.20; OCH_3 31.97.)

Oxidation of the above substance to V: The methyl ether (0.38 g) was suspended in aqueous sodium carbonate (10 ml, 2 *N*) and heated on a water-bath. Aqueous potassium permanganate (28 ml, 2.5 %) was added in small portions with mechanical stirring. Excess permanganate was destroyed with a few drops of ethanol. The filtered solution was acidified and concentrated when a small amount of yellow needles separated. M. p. 266° (Kofler bench), mixed m. p. with an authentic sample of 2,5-dimethoxyterephthalic acid showed no depression.

Ozonization of ethyl 2,6-dimethylbenzo [1.2-b, 4.5-b']-difuran-3,7 dicarboxylate (III, R = $COOC_2H_5$)

III (R = $COOC_2H_5$) (4 g) was suspended in ethyl acetate (200 ml) and ozonized at room temperature with 2.9 % ozone at a rate of 30 lh^{-1} in 80 minutes. After 60 minutes the ester had dissolved. The solvent was evaporated *in vacuo* at 20° and the resulting mixture of a yellowish-brown syrup and white crystals was treated with a small amount of ethanol. The undissolved white crystals (1.2 g) were recrystallized from ethanol. M. p. 132–134° dec. with evolution of gas. (Found: C 50.69; H 4.36; act.O 7.34. Calc. for $C_{16}H_{16}O_{12}$ (426): C 50.72; H 4.26; act.O [7] 7.51.)

0.229 g subst. in acetone (11.97 g) $\triangle t = 0.08°$ M = 409

Hydrolysis of the above substance (VI, R = $COOC_2H_5$): The ozonide (1.62 g) was boiled with aqueous sodium hydroxide (40 ml, 1 *N*) for 30 minutes. After acidifying the dark solution, the separated yellow substance (0.65 g) was recrystallized from water (charcoal). The yellow needles decomposed above 300°. (Found: C 48.61; H 3.04; Equiv. wt. 97 (0.1 *N* NaOH, phenolphthalein). Calc. for $C_8H_6O_6$: C 48.51; H 3.05; Equiv. wt. for dibasic acid 99.)

$C_8H_6O_6$ was methylated in the usual way with methyl sulphate. The resulting ether had, after recrystallization from water (charcoal), m. p. 270° (Kofler bench). Mixed melting point with an authentic sample of 2,5-dimethoxyterephthalic acid (of m. p. 266°): 268°.

Isolation of the monozonide (VII): The mother liquor from VI (R = $COOC_2H_5$) was left in an open dish for some days when yellow crystals separated (1.0 g). After recrystallization from dilute ethanol they had m. p. 115° dec. with evolution of gas. (Found: C 57.15; N 4.62; act.O 4.10; 0.406 g subst. in 9.59 g acetone, $\triangle t = 0.19°$; M = 381. Calc. for $C_{18}H_{18}O_9$ (378): C 57.17; H 4.80; act.O[7] 4.23.)

Hydrolysis of VII: VII (0.92 g) was treated with aqueous sodium hydroxide as described for VI (R = $COOC_2H_5$). The product (0.42 g) was recrystallized from ethanol (charcoal) and had m. p. 312–313° dec. (Found: C 55.97; H 3.52; Equiv. wt. 115. Calc. for $C_{11}H_8O_6$: C 55.93; H 3.41; Equiv. wt. for dibasic acid 118.)

Methyl ester of VIII: VIII (0.5 g) was esterified by means of diazomethane (from 1.8 g of nitrosomethylurea) and the product (0.55 g) recrystallized from ethanol. Yellow needles. M. p. 176°. Found: C 59.05; H 4.72; OCH_3 23.06. Calc. for $C_{13}H_{12}O_6$: C 59.10; H 4.58; OCH_3 23.49.)

Ozonization of [2,6-dimethyl-3,7-diacetylbenzo [1.2-b, 4.5-b']-difuran (III, R = $COCH_3$)

The benzodifuran (4.5 g) was suspended in ethyl acetate (200 ml) and ozonized at room temperature with 1.8 % ozone in 160 minutes. After evaporation of the solvent *in vacuo* the resulting yellow syrup was dissolved in a small amount of ethanol. This solution was

concentrated somewhat and placed in a refrigerator for several days when a light yellow substance separated (79 mg). Could not be recrystallized as it easily decomposed. No melting point. At 155° (Kofler-bench) it decrepitated instantaneously, at lower temperatures a slow decomposition took place. The crude product was used for a determination of the contents of active oxygen. (Found: act.O 7.7. Calc. for $C_{16}H_{14}O_{10}$: act.O 8.7.)

Hydrolysis of VI (R = $COCH_3$): The ozonide (90 mg) was heated with sodium carbonate (5 ml, 1 *N*) for a few minutes. On acidifying yellow needles separated (45 mg) which, recrystallized from water, decomposed at *ca.* 330°. (Found: Equiv. wt. 98. Calc. for $C_6H_4O_2$ $(COOH)_2$: Equiv.wt. 99.)

2,5-Diacetoxyterephthalic acid (IX): The syrupy mother liquor from VI (R = $COCH_3$) was concentrated further and placed in a refrigerator when a white substance separated (400 mg). Recrystallized from benzene-ethanol it decomposed at ca. 260°. (Found: C 50.98; H 3.67. Calc. for $C_{12}H_{10}O_8$: C 51.07; H 3.57.)

Hydrolysis of IX: IX (100 mg) was boiled with aqueous sodium carbonate (10 ml, 1 *N*) for a few minutes. The solution which had become yellow was acidified and yellow needles separated (75 mg). Recrystallized from water they decomposed at 335°. (Found: Equiv.wt. 101. Calc. for $C_6H_4O_2(COOH)_2$: Equiv.wt. 99.)

Methyl ester of IX: IX (230 mg) was added to an ethereal solution of diazomethane. A white substance separated which recrystallized from ethanol had m. p. 166—168°. Mixed m. p. with an authentic sample of 2,6-diacetoxyterephthalic acid dimethyl ester showed no depression. (Found: C 54.10; H 4.60. Calc. for $C_{14}H_{14}O_8$: C 54.19; H 4.54.)

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Infrared Absorption Spectra of α and β Monodeutero, and α,α' Dideutero Furan Vapours. Heat Capacity and Entropy of Furan

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The infrared absorption spectra of gaseous α and β monodeutero, and α,α'dideutero furan have been recorded in the 400—3 300 cm^{-1} region on a Beckmann I.R.3 instrument using KBr, NaCl, and LiF optics. Values for the fundamental vibrational frequencies were found or proposed in all three cases. A list of the normal vibration frequencies of furan is presented. Values of the heat capacity and the entropy are discussed.

In connection with microwave work on the deuterated furans [1] infrared absorption curves of three isotopic species were taken primarily as a check of their purity. A study of the literature on the infrared absorption of ordinary furan [2] revealed, however, that a complete vibrational assignment has not yet been given for this molecule. Therefore, a more thorough study of the spectra of the deuterated derivatives was undertaken in the hope that the problem of finding the normal vibration frequencies of furan might be solved or considerably clarified. Since only comparatively small samples were at disposal (0.5—1.5 g) which furthermore had to be conserved for conversion to deuterated pyrroles we had to omit to take infrared and Raman spectra of *liquid* samples. This disadvantage is to a large extent compensated for by the fact that for furan proper both infrared (*e. g.* this investigation) and Raman spectra [3] of the liquid have been reported.

EXPERIMENTAL

A detailed description of the preparations has been given elsewhere [1]. Infrared spectra of the vapours at 30° C and p = 50 and 350 mm Hg in each case were measured in an absorption cell 10 cm in length. The spectroscope was a Beckmann I.R.3 instrument (effective slit-width 2—5 cm^{-1}). In Table 1 observed peak frequencies and important minimum points with their corresponding optical densities are given for furan (reinvestigated here) and three deuterated species. The frequencies are thought to be good to ± 1 cm^{-1} absolutely, somewhat better "internally".

Table 1. Observed spectra of gaseous furan, α and β deutero furan, and α,α'dideutero furan in the 400—1 800 cm^{-1} region. Frequencies (F) in cm^{-1}. D = optical density at about 50 mm pressure in a 10 cm gas-cell at 30° C. Lines, thought to originate from isotopic impurities, have been omitted.

Furan		α,α'deutero furan		α deutero furan		β deutero furan	
F	*D*	*F*	*D*	*F*	*D*	*F*	*D*
585 **	0.27	490	0.42	498	0.26	501	0.17
601	0.64	505	0.85	515	0.56		
621	0.41	524	0.62	531	0.38	531	0.18
						547	0.42
725	1.0	550	0.09	585	0.11	568	0.22
744	1.5			600	0.37		
765	1.3	569	0.03	618	0.17	586	0.27
		581	0.03			601	0.60
838	0.03	586	0.03	657	0.08	620	0.45
862	0.45	630	0.07	678	0.07	630	0.43
871	0.54					646	0.48
874 *	0.38	646	0.13	699	0.11	658	0.23
881	0.55						
		667	0.29	721	0.70	667	0.35
984	1.1			738	1.3		
995	0.90	658	0.22	756	1.05	698	0.28
1 005	1.2	678	0.50				
		698	0.28	798	0.66	715	0.35
1 057	0.33			805	0.52	729	0.85
1 067	0.35	698	0.28	815	0.95	750	0.69
1 079	0.34						
ca. 1 140	0.02	751	0.27	815	0.95	750	0.69
1 168	0.59	779	0.80	858	0.10	780	0.39
1 178	0.60	782 *	0.49	866	0.09	799	0.90
1 181 *	0.49	785	0.72	879	0.10	818	0.54
1 184	0.64						
1 192	0.65	779	0.80	860	0.14	818	0.54
		798	1.5			829	0.43
1 259	0.01	815	0.64	903	0.08	837	0.20
1 268 *	0.01			912	0.07		
1 279	0.01	829	0.38	922	0.05	836	0.20
						849	0.30
1 374	0.11	886	0.10	995	0.80	864	0.36
1 384	0.10	904	0.20	1 002	0.80		
1 396	0.10	919	0.09	1 005 *	0.60	874	0.80
				1 016	0.85	886	0.60
ca. 1 460	0.23	1 005	0.45			897	0.54
		1 016	0.50	1 075	0.30		
1 478	0.58	1 019 *	0.30	1 089	0.30	1 010	0.56
1 490	0.50	1 028	0.45	1 099	0.33	1 021	0.50
1 500	0.46					1 032	0.52

* Intensity minimum in the top of a "B"-type band.

** Peak frequencies joined by ⎫ are thought to belong to one and the same band.

Furan		α,α′deutero furan		α deutero furan		β deutero furan	
F	*D*	*F*	*D*	*F*	*D*	*F*	*D*
		1 074	0.59	1 166	0.48		
1 577	0.39	1 077 *	0.37	1 169 *	0.40	1 050	0.40
1 586 *	0.30	1 088	0.57	1 174	0.52	1 061	0.43
1 591	0.31					1 074	0.45
		ca. 1 162	0.14	1 219	0.23		
ca. 1 700	0.05			1 230 *	0.17	1 121	0.04
		1 202	0.44	1 238	0.22		
1 713	0.09	1 212	0.43			1 160	0.56
1 729 *	0.03	1 224	0.47	1 306	0.04	1 171 *	0.50
1 735	0.07			1 314	0.05	1 180	0.56
		ca. 1 282	0.05	1 317	0.03		
				1 327	0.05	1 213–62	0.03
		1 306	0.09				
		1 317	0.10	1 363	0.06	1 283	0.01
		1 330	0.09	1 372	0.06	1 295	0.01
				1 382	0.07	1 307	0.02
		1 369	0.04				
				1 447	0.51	1 356	0.07
		1 420	0.60	1 460	0.61	1 367	0.07
		1 434 *	0.54	1 466	0.64	1 378	0.06
		1 438	0.80				
				1 542	0.17	1 438	0.17
		1 500	0.05	1 550	0.13	1 444	0.20
				1 562	0.18	1 457	0.25
		1 541	0.04				
		1 550 *	0.03	1 594	0.09	1 471	0.45
		1 561	0.04	1 598	0.08	1 483	0.42
				1 608	0.10	1 496	0.42
		1 587	0.08	1 626	0.04	*ca.* 1 529	0.15
		1 595 *	0.06	1 636	0.04		
		1 603	0.07	1 646	0.03	1 568	0.18
						1 577	0.22
		1 637	0.03	1 664	0.07	1 589	0.25
		1 645	0.03	1 671	0.05		
		1 653	0.03	1 684	0.08	1 637–72	0.01
		1 689	0.09	1 712	0.06	1 687	0.04
		1 698 *	0.08	1 724	0.04	1 696 *	0.03
		1 709	0.11	1 731	0.06	1 709	0.04
		ca. 1 735	0.11				

* Intensitity minimum in the top of a "B"-type band.

** Peak frequencies joined by ⎫ are thought to belong to one and the same band.

DISCUSSION OF SPECTRA

Microwave and electron-scattering experiments [1] have definitely shown that furan is planar with C_{2v}-symmetry. Unlike ten years ago one need no longer discuss the structural problem by means of infrared and Raman data [2], but one may at once state that the normal vibrations of furan are distributed according to their symmetry as summarized in Table 2. This table also surveys the direction of the electrical moment changes. The molecule is thought of as being placed in a Cartesian co-ordinate system which has its origin in the molecular centre of mass. The y-axis is perpendicular to the plane of the molecule. The z-axis lies in the molecular plane and passes through the oxygen atom. The furan selection rules also apply to the α,α'species while the situation for α and β deutero furan has been sketched in Table 3.

It is also convenient to summarize the number of "ring"-, hydrogen bending, and hydrogen stretching frequencies (Table 4, which also gives the planar or non-planar character of the modes).

Table 2. Symmetry properties of normal vibrations and selection rules for furan and α,α'dideutero furan. C_{2v}-symmetry.

Symmetry classes	Response to symm. elements		Electric moment changes parallel to	Non-genuine vibrations T = translation R = rotation	Selection rules for infrared	Number of normal vibrations	
	C_2^z	σ_y				Total	below 1 600 cm^{-1}
A_1	+	+	z	T_z	Active	8	6
B_1	−	+	x	T_x R_y	»	7	5
A_2	+	−	No changes	R_z	Inactive	3	3
B_2	−	−	y	T_y R_x	Active	3	3
				Number of normal vibrations		21	17

Table 3. Symmetry properties of normal vibrations and selection rules for α and β monodeutero furan. C_s-symmetry.

Symmetry classes	Response to symm. elements σ_y	Electric moment changes parallel to	Non-genuine vibrations T = translation R = rotation	Selection rules for infrared	Number of normal vibrations	
					total	below 1 600 cm^{-1}
A′	+	x and z	T_x, T_z, R_y	Active	15	11
A″	−	y	R_x, R_z, T_y	»	6	6
			Number of normal vibrations		21	17

Table 4. Rough geometrical characterization of the normal vibrations. Planar (+); out of plane (−).

		σ_y	Ring vibration	Hydrogen bending vibration	Hydrogen stretching vibration
Furan and α,α'dideutero furan	A_1	+	4	2	2
	B_1	+	3	2	2
	A_2	−	1	2	0
	B_2	−	1	2	0
α and β deutero furan	A'	+	7	4	4
	A''	−	2	4	0

The furan spectrum in the 400—1 600 cm^{-1} region

The Raman spectrum of liquid furan has six polarized lines at 724, 986, 1 061, 1 137, 1 380, and 1 483 cm^{-1}. This strongly suggests that the *six A_1-class vibrations below 1 600 cm^{-1}* should be found in the infrared spectrum close to these frequencies, but directly this cannot be verified from the observations. The lines to look for must be of the so-called "A"-type bands (following Badger and Zumwalt's notation [4]), which possess a central peak flanked by two side-bands each with a more or less pronounced maximum. For furan, the asymmetry parameter $\varkappa$ ("S" in the notation of Ref.[4]) is 0.916[1] while ϱ (= $(A-C)/B$, where A, B, and C are rotational constants) is 0.516. Badger and Zumwalt's $x = \pi(\nu-\nu_0)(2B/kT)^{0.5}$ becomes $(\nu-\nu_0)/16$ cm^{-1} at 30° C where $\nu-\nu_0$ is the frequency difference between a point in the "A"-band (frequency ν) and the band centre ν_0. Extrapolation from the curves in Fig. 2, Ref.[4], gives $\nu \sim 0.70$ for the *maxima* of the side-branches, so that here $\nu-\nu_0 \sim 10$ cm^{-1}. The separation of the "A"-type side branch maxima is therefore about 20 cm^{-1}. — Our recorded infrared absorption curve for gaseous furan has "A"-type bands with centres at 995, 1 067, 1 384, and 1 490 cm^{-1} with corresponding side-band separations 21, 22, 22, and 23 cm^{-1}. No immediate evidence for infrared bands near 724 and 1 137 exists but an infrared band near 724 can easily be hidden by the intense B_2-fundamental which has its band centre at 744. B_2-class vibrations are of the "C"-type bands, easily recognizable by their pronounced Q-branch and the accompanying P and R branches. For such transitions there is approximate validity of the relation [5]

$$\Delta\nu_{P,R} = \frac{S}{\pi}\sqrt{\frac{2\,kT}{I_a+I_b}} \qquad \log_{10} S = \frac{0.721}{\left(\frac{I_a+I_b}{2\,I_c}+3\right)^{1.13}}$$

(where $\Delta\nu_{P,R}$ is the frequency separation of the P and R branch maxima, $I_a < I_b < I_c$ are the principal moments of inertia) because furan is close to an oblate, symmetric top (I_a close to I_b). Using the correct values of I_a *etc.*[1] one calculates $\Delta\nu_{P,R} = 34$ cm^{-1}. Fortunately, this prediction may be tested on a B_2-band, which is certainly undisturbed by other bands due to its isolated position, the band with its centre at 601 cm^{-1}. Its $\Delta\nu_{P,R}$ is found to be 36 ± 1

cm^{-1}. Now, for the B_2-band at 744 we find that $\Delta\nu_{P,R} = 40 \pm 1$ cm^{-1}. Since the two $\Delta\nu_{Q,R}$ of the two B_2-bands practically agree it must be the two $\Delta\nu_{P,Q}$'s which differ by about 4 cm^{-1} so that the presence of some disturbance in the P-branch of the 744-band may be postulated. By comparison of the intensity distribution of the two B_2-bands (which should show marked similarities) it is seen that the P-branch of the 744-band is somewhat more intense than expected. *Together*, the arguments presented in favour of speaking of an "observed" line near 724 cm^{-1} (the maximum of the P-branch) seem almost conclusive. It is confirmed by the study of isotopic species.

The other "missing" A_1-fundamental also has an unfavourable anticipated position in the "foot" of the very strong B_1-fundamental at 1 181. This band is of the "B"-type which for furan has less pronounced maxima (if any) in the side-branches so that a check as performed above is impossible. However, an intensity irregularity is clearly visible in the 1 120—1 145 region. Features from the spectra of the deuterated species help to show that there is a fundamental here.

The *five fundamentals of the B_1-class below 1 600 cm^{-1}* are to be "B"-type bands. For furan, these will have a relative intensity *minimum* at the centre flanked by two closely spaced maxima (*separation* 3—5 cm^{-1} which, due to perturbations, may not be observable so that a single *maximum* appears). A "B"-type band terminates in side-bands with or without maxima which, if observable, must be separated about 20 cm^{-1}. Bands with a reasonable resemblance to this description are found at 1 181 (1 171), 1 268 (1 270), and 1 586 (?) (numbers in parentheses are observed, depolarized Raman shifts). A strong, observed band at 874 has some resemblance to, *e. g.*, the A_1-band at 1 067 (although it is far less symmetrical). But since the corresponding Raman line is depolarized and since 874 cannot be a "C"-type band (the bands of the B_2-class) 874 is excluded from other classes than B_1. Data from the isotopic species verify this. — Thompson [2] has questioned whether 1 181 belongs to the B_1-class. In our records of gaseous and liquid furan it is easy to see that the line is disturbed by something in the "foot" (as already mentioned) and something in the "tail", but its central part looks to us as if it were a "B"-type line.

Two out of five B_1-type vibrations at 874, 1 181, 1 268, 1 586, and an unknown frequency are expected to be hydrogen bending vibrations (Table 4). These two can be any of the three at 874, 1 181, and 1 268. Probably we are still lacking one "ring"-vibration. In their paper on pyrrole and deuterated species Lord and Miller [6] found that two "ring"-vibrations in the B_1-class had to be located at 1 418 and 1 530. Obviously, our band at 1 586 corresponds to the pyrrole band at 1 530. Therefore, we may look for another "ring"-vibration in the vicinity of 1 470 cm^{-1}. Actually, a band is observed in the "foot" of the band at 1 490. Its approximate location is 1 455 cm^{-1}. A depolarized Raman line has been observed by Reitz [3] at 1 455. Data from the deuterated species seem to confirm our choice.

Two of *the three B_2-bands* are easily found due to their pronounced "C"-type structure, the bands at 601 and 744 cm^{-1}. As already mentioned by Thompson [2] it might be the intense Q-branch of the third B_2-band that peeps up at 838 in the "foot" of the strong B_1-fundamental at 874. A depolarized Raman line [3] has been observed at 839 cm^{-1}.

The three A_2-fundamentals are not active in the infrared but should appear in the Raman spectrum. An observed Raman line at 1 034 cm^{-1} is naturally assigned to the A_2-class. Confirmation is obtained by considering the infrared spectrum of *liquid* furan. Here, the selection rules may break down and actually a small, but distinct line is observed at 1 030. Roughly speaking, the two remaining frequencies in the A_2-class should lie in the 500—700 cm^{-1} region. An otherwise unexplained line at 1 090 cm^{-1} in the spectrum of liquid furan might be the first overtone of one of the A_2-fundamentals (at *ca.* 545 cm^{-1}). Guthrie *et al.*[7] have suggested that the "B"-type band at 1 268 is the first overtone of one of the A_2-fundamentals, and not a fundamental as suggested by us. Therefore, the third A_2-fundamental could lie at about 640 cm^{-1}. We want to maintain that 1 268 is a fundamental (of the B_1-class) since this gives a natural explanation of the very strong bands at 1 212 and 1 230 in the α and α,α' deuterated species. Of course, this does not exclude that two lines coincide at 1 268 in the spectrum of furan. Indeed, product rule calculations to be given later in this paper indicate that A_2-fundamentals near 1 030, 660, and 550 are highly probable. An observed, weak Raman line at 1 324 cm^{-1} could be the first overtone of a 660-fundamental.

The furan spectrum in the 1 600—3 300 cm^{-1} region

Two overtones of definite "A"- or "B"-type structure are observed at 1 700 and 1 729. Hydrogen stretching frequencies are located at 3 124 and 3 163. Inbetween, quite a few bands (weak) appear but their contours are disappointingly complex and difficult to interpret. We shall, therefore, renounce from an attempt to utilize this part of the absorption curve.

Bands at 1 700 and 1 729 may be interpreted as first overtones or one of the combinations $A_1 + B_1$, $A_1 + A_1$, $B_1 + B_1$, $A_2 + A_2$, $B_2 + B_2$, or $A_2 + B_2$. If there are first overtones at 1 700 and 1 729 there should be fundamentals at 850 and 865. 1 729 may, therefore, be interpreted as $2 \times 874(B_1) = 1\,748$, being 1 % anharmonic. 1 700 we may explain as $720(A_1) + 995(A_1) = 1\,715$, also being 1 % anharmonic. These facts leave no room for well-founded speculations as to the magnitude of the missing A_2-frequencies.

The fundamentals finally adopted in this paper for furan are given in Table 7. These values have consequences to be discussed for a comparison between the theoretical and the experimental C_p-curve and the entropy of furan recently studied by Guthrie *et al.*[7].

The α,α'dideutero furan spectrum

This compound has the same symmetry species as furan. Its $\varkappa$ is 0.632 [1] $\varrho = 0.582$. Badger and Zumwalt's $x = (\nu - \nu_0)/15$ $cm^{-1} = 0.75$. The "A"-type band side branch separation is, therefore, about 22 cm^{-1}. For "B"-type bands it is the same (if discernable). For "C"-type bands one calculates $\Delta\nu_{P,R} = 33$ cm^{-1}. Since the least moment of inertia here is about the x-axis (in furan it is about the z-axiz) we know that class A_1-vibrations must be of the "B"-type, class B_1-vibrations of the "A"-type, and class B_2-vibrations of the "C"-type while class A_2-vibrations are inactive in the infrared spectrum.

Selection of the fundamentals given in Table 7 is easily carried through under due regard to the intensity, the shape, and the position of the bands except in the case of the A_1-vibration at 698 and the B_1-vibration at 667. The peaks observed at these frequencies lie in the P and R branch of the C-type band at 678. They show up quite distinctly inspite of this but it is impossible to say anything about their band contour. Justification for their choice as fundamentals may, however, be found in the circumstance that the calculated product-rule ratios (Table 5) for the two symmetry classes involved fit tolerably with the anticipated.

Table 5. Calculated and experimental product-rule ratios. $\overset{n}{\Pi}(a,K)$ *means the product of the n normal vibration frequencies in the symmetry class K for the a derivative. The corresponding symbol for furan itself is* $\overset{n}{\Pi}(o,K)$.

	Product rule ratios		
	Calculated	Observed	Deviation %
$\frac{\overset{8}{\Pi}(o, A_1)}{\overset{8}{\Pi}(aa', A_1)}$	1.971	2.094	+ 6.2
$\frac{\overset{7}{\Pi}(o, B_1)}{\overset{7}{\Pi}(aa', B_1)}$	1.888	1.932	+ 2.3
$\frac{\overset{3}{\Pi}(o, A_2)}{\overset{3}{\Pi}(aa', A_2)}$	1.355		
$\frac{\overset{3}{\Pi}(o, B_2)}{\overset{3}{\Pi}(aa', B_2)}$	1.364	1.371	+ 0.5
$\frac{\overset{15}{\Pi}(o, A_1 + B_1)}{\overset{15}{\Pi}(a, A')}$	1.929	2.152	+ 11.6
$\frac{\overset{15}{\Pi}(o, A_1 + B_1)}{\overset{15}{\Pi}(\beta, A')}$	1.925	2.135	+ 10.9
$\frac{\overset{6}{\Pi}(o, A_2 + B_2)}{\overset{6}{\Pi}(a, A'')}$	1.345	1.334	− 0.8
$\frac{\overset{6}{\Pi}(o, A_2 + B_2)}{\overset{6}{\Pi}(\beta, A'')}$	1.345	1.334	− 0.8

An interpretation of combination- and overtones is given in Table 6. Only in one case it is absolutely necessary to assume that an A_2-class vibration is involved, namely the "A"-type band at 1 645. This band might therefore be the first overtone of an A_2-fundamental at about 830. It can also be one of the combinations A_2+B_2 which gives rise to the possibilities 847, 967, and 1 140. Since the two last are too high (compare the spectra of the mono-deuterated derivatives) an A_2-fundamental may lie at 847 cm^{-1}. We shall see later that the existence of an A_2-fundamental near 830-50 agrees well with data from the monodeuterated species.

The α deutero furan spectrum

Here, we have only two symmetry-classes, A′ (planar vibrations, "A"- or "B"-type bands) and A″ (out-of-plane vibrations, "C"-type bands). $\varkappa$ is 0.733. $\varrho = 0.557$ [1]. Again, the "A"- and "B"-type band side branch separation is about 22 cm^{-1} while the $\Delta\nu_{P,R}$ for "C"-type bands is 33 cm^{-1}.

As for the α,α'-derivatives assignment of the majority of the fundamentals is pretty straightforward. In the A′-class 1 598 was preferred to 1 550 in order to obtain good agreement with the product-rule assuming that the band at 699 is a fundamental. 657 was preferred to 678 as a fundamental since it gives the better product-rule fit when the relation $\left[\prod^{6}(\alpha,A'')\right]/\left[\prod^{6}(\beta,A'')\right] \sim 1.000$ is used (for the β-derivative the six frequencies of the A″-class can be assigned without discussion).

Combination- and overtones are interpreted in Table 6.

The β deutero furan spectrum

The fundamentals adopted and the interpretation of the overtones *etc.* are given in Tables 7 and 6. The line at 698 has not been assigned to the A′-class because of its pronounced "C"-type band structure. We have, therefore, assumed that the missing A′-fundamental is hidden in the strong band at 729.

FINAL ASSIGNMENT OF FURAN FUNDAMENTALS

As a result of the fore-going discussion we are now able to state that probably the fundamentals of furan and the deuterated species here considered are located as given in Table 7.

The two low-frequency A_2-class fundamentals of furan have been estimated to 550 and 660. This is reasonable in view of the position of the corresponding frequencies in the mono-deuterated species. If they were chosen to be higher the product-rule ratios which control their magnitude would deviate unfavourably from the predicted values.

We might finally try to guess the magnitude of the three missing A_2-fundamentals in the α,α'-derivative spectrum. As already mentioned there is some reason to believe that the fundamental of highest frequency is

Table 6. Observed infrared absorption lines explained as combination- and overtones.

	Position	Type	Interpretation
Furan	1 700	A	$720(A_1) + 995(A_1) = 1\,715(A_1)$
	1 729	A	$2 \times 874(B_1) = 1\,748(A_1)$
α,α'dideutero furan	550	A	$1\,212(B_1) - 667(B_1) = 545(A_1)$
	581	A	$1\,369(B_1) - 782(A_1) = 587(B_1)$
	630	C	$1\,317(A_1) - 678(B_2) = 639(B_2)$
	646	?	$1\,434(A_1) - 782(A_1) = 652(A_1)$
	751	C	$1\,434(A_1) - 678(B_2) = 756(B_2)$
	829	?	$1\,550(B_1) - 698(A_1) = 852(B_1)$
	904	C	$1\,434(A_1) - 505(B_2) = 929(B_2)$
	1 282	A	$505(B_2) + 798(B_2) = 1\,303(A_1)$
	1 500	?	$698(A_1) + 798(B_2) = 1\,496(B_2)$
	1 595	A	$2 \times 798(B_2) = 1\,596(A_1)$
	1 645	A	2×830(ass. A_2-fund.); $798(B_2) + 847$ (ass.A_2)
	1 698	A	$1\,019(A_1) + 667(B_1) = 1\,686(B_1)$
	1 735	?	$1\,077(A_1) + 667(B_1) = 1\,744(B_1)$
α deutero furan	678	C	$1\,550(A') - 860(A'') = 690(A'')$
	866	A	$1\,550(A') - 699(A') = 851(A')$
	1 550	A	$738(A'') + 815(A'') = 1\,553(A')$
	1 636	A	$2 \times 815(A'') = 1\,630(A')$
	1 671	A	$815(A'') + 860(A'') = 1\,675(A')$
	1 724	A	$2 \times 860(A'') = 1\,720(A')$
β deutero furan	501	C	$1\,061(A') - 547(A'') = 514$
	667	C	$1\,483(A') - 799(A'') = 684$
	698	C	$1\,295(A') - 601(A'') = 694$
	750	C	$1\,295(A') - 547(A'') = 748$
	1 121	?	$547(A'') + 601(A'') = 1\,148(A')$
	1 444	A	$2 \times 720(A') = 1\,440(A')$
	1 529	A	$729(A'') + 799(A'') = 1\,528(A')$
	1 637-72	A	$849(A'') + 799(A'') = 1\,648(A')$
	1 696	A	$2 \times 849(A'') = 1\,698(A')$

situated in the 830—850 cm^{-1} region. From this and the product-rule it follows that the product of the remaining frequencies must be $(3.324\text{-}3.246) \times 10^5$. Since it is seen by comparison with the frequencies of the mono-deuterated species that one of the remaining frequencies may lie near 500 cm^{-1} it is a consequence that the third lie in the region 665—650. A value near 650 cm^{-1} fits nicely into the whole picture.

THERMODYNAMIC PROPERTIES OF FURAN

In their great paper on furan Guthrie *et al.*[7] have compared precalculated C_p- and entropy-values with experimental determinations. Guthrie has substantially chosen the same set of fundamental vibrational frequencies as ours with the exception of three. Instead of our frequencies at 550, 1 268, and 1 460 cm^{-1} Guthrie selected empirically frequencies at 700, 1 000, and

Table 7. Position of normal vibration frequencies for furan, α and β monodeutero furan, and α,α'dideutero furan. Frequencies in cm^{-1}.

	Furan	α-derivative		β-derivative	α,α'-derivative	
A_1	*ca.* 720	699	A'	*ca.* 720	698	A_1
	995	912		886	782	
	1 067	1 005		1 021	1 019	
	ca. 1 140	1 089		1 061	1 077	
	1 384	1 317		1 295	1 317	
	1 490	1 456		1 483	1 434	
	3 124	3 128		3 130	2 366	
	3 124	3 128		3 130	3 129	
B_1	874	805		829	667	B_2
	1 181	1 169		1 171	*ca.* 1 162	
	1 268	1 230		1 242	1 212	
	ca. 1 460	1 372		1 367	*ca.* 1 369	
	1 586	1 598		1 577	1 550	
	3 163	2 349		2 332	2 412	
	3 163	3 166		3 161	3 265	
A_2	550	515	A''	547	?	A_2
	660	657		646	?	
	1 030	860		849	?	
B_2	601	600		601	505	B_2
	744	738		729	678	
	838	815		799	798	

1 300 which brought the calculated thermodynamic functions (C_p° and S°) into best possible agreement with the experimental calorimetric data. In Table 8 the C_p-values found by Guthrie are compared with the ones, calculated by him and with the values calculated by us, using the same rigid-rotor, harmonic-oscillator approximation.

The agreement with experiment directly aimed at by Guthrie is definitely better than ours, especially at higher temperatures. Here, the rigid-rotor, harmonic-oscillator approximation is definitely too poor, since the assumption of harmonic vibrations is equivalent to assuming a too widely spaced

Table 8. Comparison between experimental and calculated C_p° values for furan (cal. deg.$^{-1}$mole^{-1}).

T. °K	317.25	358.20	402.20	449.20	487.20
C°_p (experim.)	16.80*W*.03	19.15	21.51	23.80	25.45
Calc. (Guthrie)	16.74	19.02	21.31	23.55	25.20
Calc. (B—B—H)	16.73	18.92	21.14	23.32	24.94

energy level pattern. It is not difficult to correct approximately for this at *low* temperature, say 317.25° K (Table 8). If roughly all observed frequencies are 1 % anharmonic, a "reduced" harmonic oscillator energy pattern may be introduced as shown in Fig. 1.

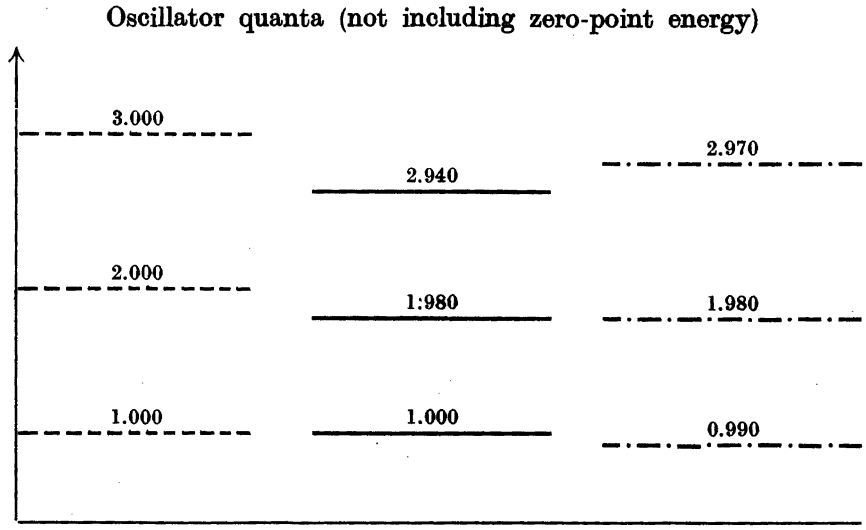

Fig. 1. Energy pattern for usual "harmonic" oscillator approximation (— — — —), for assumed 1 % anharmonicity (————), and for "reduced" harmonic oscillator (— · — · — · —). One-dimensional example.

The "reduced" energy pattern is seen to lie lower than the first excited state, to coincide with the second, and to lie higher than the third. Since this last level is very thinly populated at room temperature the populated "reduced" energy levels must, on average, lie lower than the actual, so that a somewhat too *high* C°_p value should result from a calculation. By recalculation of the heat capacity at 317.25° K, using fundamental frequencies artificially lowered by 1 %, we calculate $C^\circ_p = 16.82$ cal. deg.$^{-1}$ mole^{-1}. This fits nicely with the experimental value (16.80). Unfortunately, this way of correcting cannot be applied at higher temperatures where the third, fourth *etc.* levels also become populated. The correction method given by Stockmeyer, Kavanagh, and Mickley [8] has been used for benzene by Scott *et al.*[9]. At nearly the same temperatures as those in Table 8 they found differences between observed and calculated C°_p values (using harmonic oscillator levels) of magnitude: 0.13; 0.17; 0.23; 0.36. Scott *et al.* showed that these differences could be identified with Stockmeyer's C_p -correction by adjustment of a single parameter. Since somewhat *smaller* differences may be expected in the case of furan, it is seen that it is hardly possible to bring our calculated C_p values in as fine a harmony with experiment as Guthrie *et al.* succeeded in obtaining. We are unable to explain the discrepancy. Guthrie *et al.* claim that "no group of (funda-

mental) frequencies including one below 600 cm^{-1} was found which gave reasonable agreement between calculated and observed thermodynamic properties". In order to agree with this statement we would have to abandon the idea of having "C"-type fundamentals in the spectra of the *mono*deuterated species at 515 and 547 cm^{-1} together with all the useful consequences for the interpretation of the whole spectral material derived from this.

In Table 9 we shall finally compare three experimental entropy values given by Guthrie *et al.*[7] with values calculated by him and his collaborators and values calculated by us, based on the new set of vibrational fundamentals.

Table 9. Comparison between experimental and statistical entropy values (in cal. deg.$^{-1}$ mole^{-1}).

T, °K	279.16	293.16	304.52
Experimental entropy	62.87±0.10	63.60	64.18
Calc. by Guthrie	62.86	63.60	64.19
» in this investig.	63.03	63.77	64.36

Our calculated entropy values are, therefore, about 0.17 entropy units too *high*. Due to experimental uncertainty the difference need not exceed 0.07 e.u. Perhaps this discrepancy can be explained by reference to Guthrie's paper. From the report of the experiments it follows that it could not be definitely excluded that furan did not form thermodynamically perfect crystals under the experimental conditions since a hysteresis in one of the transitions was noted.

A few values of the "reduced free energy" from our data are given in Table 10.

Table 10. Values of $-\left(\frac{G^\circ - H^\circ_o}{T}\right)$* *for furan in cal. deg.$^{-1}$mole^{-1}.*

T° K	$-\left(\frac{G^\circ - H^\circ_o}{T}\right)$
273.16	53.16
298.16	54.02
300	54.07
400	57.25
500	60.20
600	63.01
700	65.72
800	68.31

The values of Table 10 deviate 0.06—0.10 cal deg.$^{-1}$ mole^{-1} from those given by Guthrie *et al.*[6].

* $-\left(\frac{F^\circ - H^\circ_o}{T}\right)$ in American literature.

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Studies on the Extraction of Metal Complexes

XX. The Dissociation Constants and Partition Coefficients of 1-Nitroso-2-naphthol and 2-Nitroso-1-naphthol

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Dissociation constants, partition coefficients, solubilities and absorption spectra have been determined at 25° C for 1-nitroso-2-naphthol and 2-nitroso-1-naphthol. The two compounds have quite different properties, which are discussed. An explanation of these differences is given with reference to the infrared spectra, and to the electron distribution in naphthalene.

In our studies of the extraction of metal ions with organic reagents we have found it important to know the acid dissociation constants and partition coefficients of the organic reagents. As these constants are fundamental properties of the compounds themselves we consider it advisable to present these values separately and not together with the metal extraction data. The present work gives the measurements on 1-nitroso-2-naphthol and 2-nitroso-1-naphthol.

EXPERIMENTAL

Commercially available analytical reagents (B. D. H. and Merck) were recrystallized from alcohol (1-nitroso-2-naphthol) and from chloroform (2-nitroso-1-naphthol). All experiments were carried out at 25° C and in a medium of 0.1 *M* ionic strength. All chloroform used was alcohol-free and saturated with water. The hexone (methyl *iso*butyl ketone) was shaken with a sodium bicarbonate solution to remove acidic or basic impurities.

The potentiometric titrations on 1-nitroso-2-naphthol were carried out as previously described [1,2] using 0.01 *M* NaOH + 0.09 *M* $NaClO_4$ for the titration of 0.50 ml of 0.0991 *M* $HClO_4$ + 100 ml of 2×10^{-4} *M* 1-nitroso-2-naphthol in 0.1 *M* $NaClO_4$. The equivalent points were obtained by Gran's method of plotting $\frac{\Delta V/\Delta E}{V}$ against the volume of alkali added [3].

The spectrophotometric measurements of the dissociation constants were made in a thermostated room using a Beckman Model DU spectrophotometer with 1 cm cells. The solutions were made up of 9 ml $1-2 \times 10^{-4}$ *M* nitrosonaphthol in 0.1 *M* $NaClO_4$ + 1 ml of 0.1 *M* sodium acetate-, phosphate-, borate-perchlorate buffers. The hydrogen ion concentration, $-\log[H^+]$ of the solutions were determined with a 0.01 *M* $HClO_4$ + 0.09 *M* $NaClO_4$ buffer.

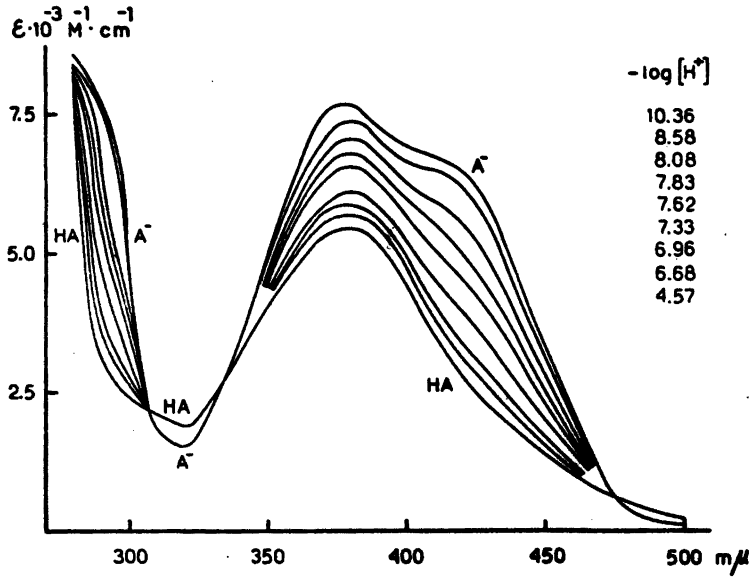


Fig. 1. Variation of the absorption spectra of 1-nitroso-2-naphthol in 0.1 M $NaClO_4$ with acidity.

The partition coefficients for a 0.1000 *M* solution of 1-nitroso-2-naphthol in chloroform or hexone and a 0.0500 *M* solution of 2-nitroso-1-naphthol were determined by shaking 10 ml of the organic phase with 9 ml of 0.1 *M* $NaClO_4$ + 1 ml of a 0.1 *M* sodium acetate-perchlorate buffer. The concentration of reagent in the aqueous phase was determined spectrophotometrically.

CALCULATION OF THE DISSOCIATION CONSTANTS FROM THE SPECTROPHOTOMETRIC DATA

Both 1-nitroso-2-naphthol and 2-nitroso-1-naphthol showed measurable shifts in their spectra in the pH range of 5—10 as may be seen in Figs. 1 and 2. These shifts are due to the dissociation of the nitrosonaphthols and to the fact that the acidic and basic forms have different absorption spectra. At some wavelengths the molar absorptivities of the two forms are the same, *i. e.* the two extinction curves intersect. These points of intersection are called isobestic points and are useful for the indication of solvent effects (*e. g.* a shift in the extinction curve of acidic form when the ionic medium is changed). By keeping the ionic medium constant at 0.1 *M* with mainly $NaClO_4$ we obtained very sharp isobestic points (Figs. 1 and 2).

In the case of the nitrosonaphthols we could determine both the absorption of the acidic from A_o at pH<5 and the absorption of the basic form A_- at pH>10. For a constant total concentration of the nitrosonaphthol the dissociation constant k_a was then obtained from the following equation:

$$k_a = [H^+] \cdot \frac{(A - A_o)}{(A_- - A)}.$$

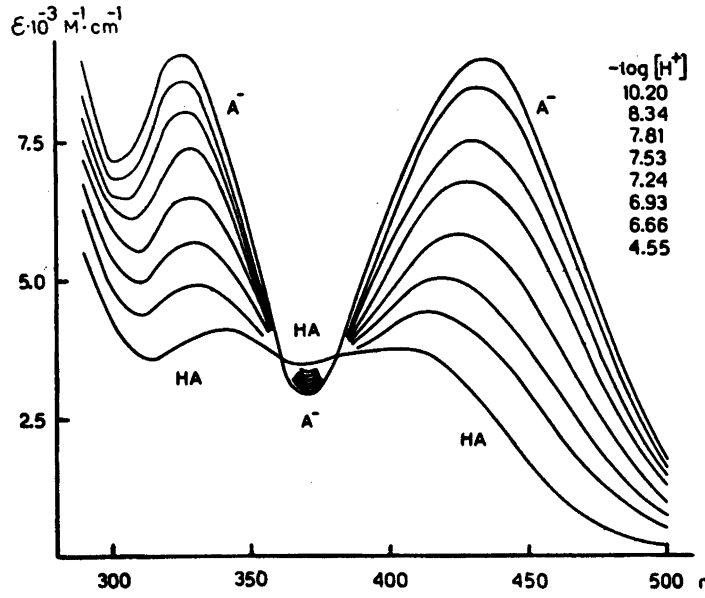


Fig. 2. Variation of the absorption spectra of 2-nitroso-1-naphthol in 0.1 M *$NaClO_4$ with acidity.*

Data for several wavelengths (Tables 1 and 2) were used for the calculation of log $(A—A_o)/(A_-—A)$. These values were then plotted against — log[H^+]. The best line with a slope equal to 1 gave pk_a at log $(A—A_o)/(A_-—A) = 0$.

The following values were obtained for the dissociation constants in 0.1 *M* $NaClO_4$:

Table 1. Spectrophotometric determination of pk_a *for 1-nitroso-2-naphthol in 0.1* M *$NaClO_4$. The absorption* A *of* 10^{-4} M *solutions at different values of* $-\log [H^+]$ *for wavelengths used in the calculation of* pk_a.

λ mμ	−log [H+]										
	1.80	3.03	4.57	6.68	6.96	7.33	7.62	7.83	8.08	8.58	10.46
290	0.322	0.322	0.317	0.363	0.400	0.470	0.567	0.612	0.667	0.767	0.778
370	0.531	0.534	0.528	0.556	0.573	0.584	0.628	0.651	0.673	0.701	0.751
380	0.550	0.548	0.545	0.567	0.584	0.612	0.656	0.678	0.706	0.739	0.767
390	0.528	0.534	0.520	0.548	0.567	0.584	0.628	0.651	0.678	0.706	0.728
400	0.446	0.445	0.448	0.478	0.500	0.528	0.573	0.600	0.628	0.667	0.695
410	0.355	0.353	0.356	0.384	0.409	0.456	0.512	0.556	0.595	0.651	0.678
420	0.281	0.281	0.279	0.317	0.341	0.400	0.461	0.503	0.556	0.617	0.651
430	0.224	0.225	0.224	0.259	0.284	0.339	0.400	0.442	0.489	0.550	0.584
440	0.176	0.178	0.183	0.203	0.222	0.270	0.316	0.348	0.384	0.431	0.459
450	0.136	0.137	0.140	0.156	0.168	0.203	0.235	0.257	0.284	0.316	0.338

Table 2. Spectrophotometric determination of pk_a *for 2-nitroso-1-naphthol in 0.1 M* $NaClO_4$. *The absorption* A *of* 10^{-4} M *solutions at different values of* $-\log [H^+]$ *for wavelengths used in the calculation of* pk_a.

λ mμ	−log [H+]									
	2.01	3.02	4.55	6.66	6.93	7.24	7.53	7.81	8.34	10.20
310	0.361	0.367	0.367	0.442	0.500	0.556	0.617	0.662	0.706	0.751
320	0.370	0.370	0.367	0.473	0.545	0.623	0.695	0.778	0.840	0.890
330	0.389	0.394	0.395	0.495	0.573	0.651	0.739	0.801	0.851	0.901
340	0.403	0.408	0.411	0.481	0.539	0.606	0.667	0.701	0.723	0.762
410	0.374	0.374	0.375	0.442	0.495	0.545	0.612	0.662	0.723	0.751
420	0.344	0.346	0.346	0.436	0.503	0.584	0.667	0.723	0.806	0.845
430	0.297	0.296	0.299	0.409	0.489	0.578	0.678	0.751	0.845	0.890
440	0.235	0.234	0.235	0.359	0.442	0.545	0.651	0.723	0.834	0.890
450	0.171	0.170	0.170	0.295	0.378	0.475	0.589	0.662	0.751	0.795
460	0.112	0.111	0.113	0.228	0.303	0.394	0.492	0.556	0.634	0.667
470	0.071	0.070	0.072	0.167	0.232	0.305	0.388	0.442	0.500	0.537
480	0.044	0.045	0.046	0.118	0.164	0.222	0.280	0.322	0.363	0.392
490	0.028	0.028	0.029	0.083	0.112	0.151	0.195	0.223	0.251	0.270

1-nitroso-2-naphthol: $pk_a = 7.63 \pm 0.02$ ($pK_a = 7.77$ *)
2-nitroso-1-naphthol: $pk_a = 7.24 \pm 0.02$ ($pK_a = 7.38$ *)

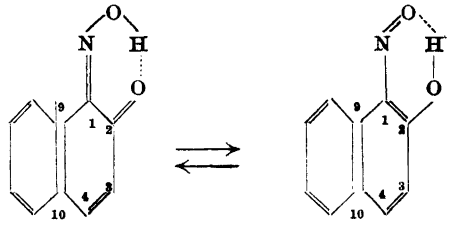
A potentiometric determination for 1-nitroso-2-naphthol gave $pk_a = 7.59 \pm 0.02$ in close agreement of the spectrophotometric value.

The dissociation constants have previously been determined by Trübsbach[5] at 25° C by conductometric measurements of dilute solutions. He obtained $K_a = 26 \times 10^{-9}$ ($pK_a = 7.59$) for both 1-nitroso-2-naphthol and 2-nitroso-1-naphthol. A result given by Wenger, Monnier and Jaccard[6] seems to be based on an erronous interpretation of a potentiometric titration.

DISCUSSION

It is quite clear from the data collected in Table 3 and from Fig. 3 that 1-nitroso-2-naphthol and 2-nitroso-1-naphthol have quite different properties.

The question arises whether these differences can be explained by a tautomeric equilibrium:



(A) (B)

* Thermodynamic values. The activity coefficients were taken from Conway[4], p. 102—3.

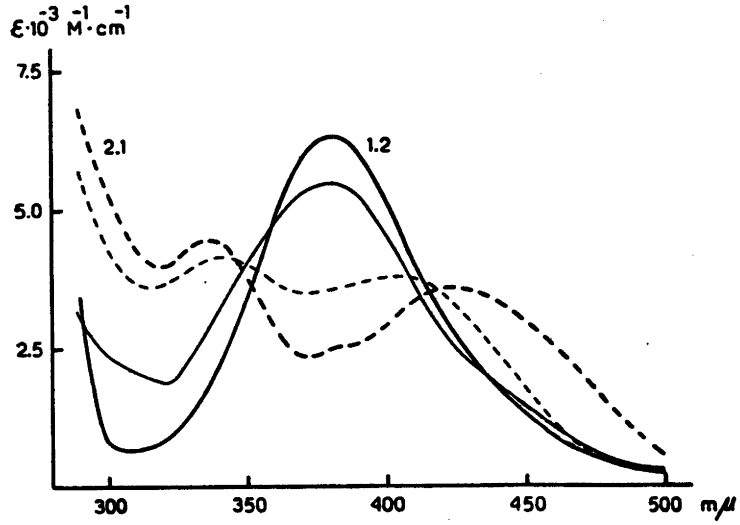


Fig. 3. Absorption spectra of the nitrosonaphthols in chloroform (thick lines) and in water (thin lines). The full curves represent 1-nitroso-2-naphthol; the dashed curves 2-nitroso-1-naphthol.

the two nitrosonaphthols having different equilibrium constants. However, evidence has been given by Sluiter [7], as well as by French and Perkins [8] and by Baltazzi [9] that the quinonoid modification (A) predominates. Infrared studies in our laboratory [10] showed that a strong intramolecular hydrogen bond is present in 1-nitroso-2-naphthol (Fig. 4). The strength of this bond is comparable with the one in acetylacetone [11], and stronger than the one in oxine and 2-nitrophenol (Fig. 4) or tropolone [11]. Furthermore the infrared spectrum showed the presence of a broad carbonyl band at 1 625 cm^{-1}. This carbonyl band is also observed in β-diketones and tropolone (*cf.* Bellamy [11], p. 123, 130). The infrared data therefore support the view that the quinonoid form (A) predominates, the length of the OH-bond being 1.10 Å. The distance from the hydrogen atom to the quinone oxygen can be estimated at 1.5 Å, which is much less than the sum of the van der Waals radii (2.6 Å).

The hydrogen bridging and chelate ring stabilisation is also supported by the fact that, according to Trübsbach [5], 1-nitroso-2-naphthol and 2-nitroso-1-naphthol are not much stronger but weaker acids than 4-nitroso-1-naphthol ($pK_a = 6.59$). The same order of acidity has been found for 2-nitro-1-naphthol and 4-nitro-1-naphthol [12]. Against this Havinga and Schors [13] have reported $pK = 8.01 \pm 0.04$ ($pK_a = 8.05$) for 4-nitroso-1-naphthol.

It is now clear from experimental data of Abrahams, Robertson and White [14] as well as from π-electron calculations of Jaffé [15] and of Klement [16] that in naphthalene the carbon atoms in positions 9 and 10 have an inductive effect which of course acts more strongly on the 1-positions than on the 2-positions. Thus the electron densities at the carbon atoms in naphthalene decrease in the order $C_9 > C_1 > C_2$.

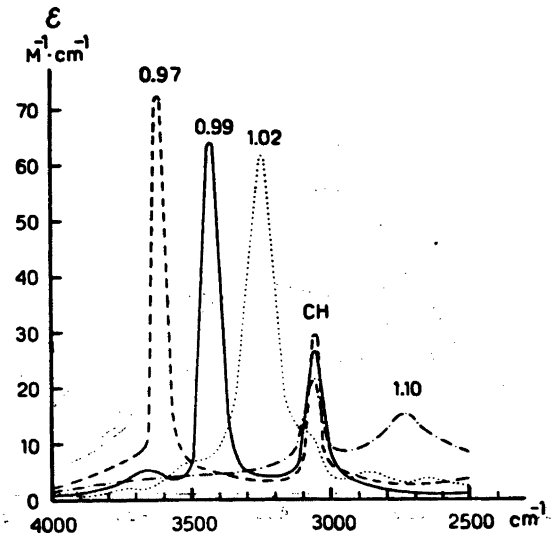
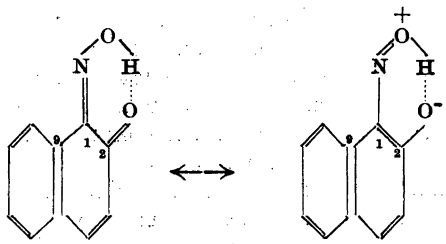


Fig. 4. Infrared absorption spectra of 1-naphthol — — — —, 8-quinolinol (oxine) ————, 2-nitrophenol . . . . . , and 1-nitroso-2-naphthol — · — · — · — in CCl_4 at 25 °C. The OH peaks correspond to different lengths of the OH bonds (in Å).

It is also clear that the bond between the 1 and 2 carbons has a higher electron density than other bonds; this would favor the contribution of structure (A') to a mesomeric state, or the tautomeric form (B).



(A) (A')

The difference in acidities of the two nitrosonaphthols may be explained by a higher inductive effect acting on the quinonoid oxygen in position 1 thus weakening the hydrogen bond and giving a stronger acid. Thus 2-nitroso-1-naphthol would be expected to be a stronger acid than 1-nitroso-2-naphthol, and this in fact is the case. Lauer [17] has shown that 1-naphthol ($pk_a = 9.85$) is a stronger acid than 2-naphthol ($pk_a = 9.93$), however, the colorimetric method with phenolphthalein used for the determination of the dissociation constants is not very accurate. In fact Kieffer and Rumpf [18] have recently obtained $pK_a = 9.23$ for 1-naphthol and 9.46 for 2-naphthol at 25° C.

Table 3. Properties of the nitrosonaphthols.

Property	1-nitroso-2-naphthol	2-nitroso-1-naphthol
Dissociation constant pk_a in 0.1 M $NaClO_4$	7.63 ± 0.02	7.24 ± 0.02
Partition coefficient log k_d between chloroform and water (I = 0.1 M)	2.97	2.11
Partition coefficient log k_d between hexone and water (I = 0.1 M)	2.55	2.23
Solubility in chloroform (aq) in moles per liter	1.35_2	0.096_4
Solubility in hexone (aq) in moles per liter	0.416	0.133
Solubility in water (pH = 4.6, I = 0.1 M)	1.06×10^{-3}	0.84×10^{-3}
Melting point °C.	112	160

Schenkel [19] has given an explanation of Lauer's results based on earlier conceptions of the electron distribution in naphthalene. However, the electron distribution is now known to be somewhat different.

Besides the spectra and dissociation constants the solubilities show a striking difference, 1-nitroso-2-naphthol being much more soluble in chloroform. In hexone, which contains an oxygen atom, the difference is not so large and in water the difference is still less. From these facts it might be concluded that the chelate ring is not so well stabilized in 2-nitroso-1-naphthol, and since this ring stabilisation lowers the acidity, 1-nitroso-2-naphthol should be a weaker acid. It therefore seems as if the inductive effect mentioned above has an effect on the resonance in the chelate ring. Infrared measurements [10] on 2-nitroso-1-naphthol were difficult because of its low solubility in CCl_4. A somewhat weaker hydrogen bond and a weaker carbonyl band were however discernable. The melting points and partition coefficients also support the conclusion that the chelate ring is more stable in 1-nitroso-2-naphthol.

The authors wish to thank the head of FOA 1, Professor Gustaf Ljunggren for his continuous interest in these metal extraction studies.

We are very grateful to Fil.lic. Lennart Larsson for valuable discussions in connection with his measurements of the infrared spectra.

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Studies on Aspartase

V. Inactivation and Reactivation of Aspartase

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The inactivation of aspartase using prolonged dialysis, electrodialysis and treatment with different forms of Amberlite IR-120 is described. Under carefully controlled conditions the partially inactivated enzyme was completely reactivated by incubation with different divalent metal ions. Ca^{++} and Mn^{++} proved to be the strongest activators even at low metal concentrations. Much higher concentrations of Ba^{++}, Sr^{++} and Mg^{++} were needed to obtain the same activity. Inactivators like Co^{++} and Pb^{++} seemed to produce a slight activation at low concentration. No activation or inactivation was obtained with Ni^{++}, Fe^{++}, and Zn^{++}.

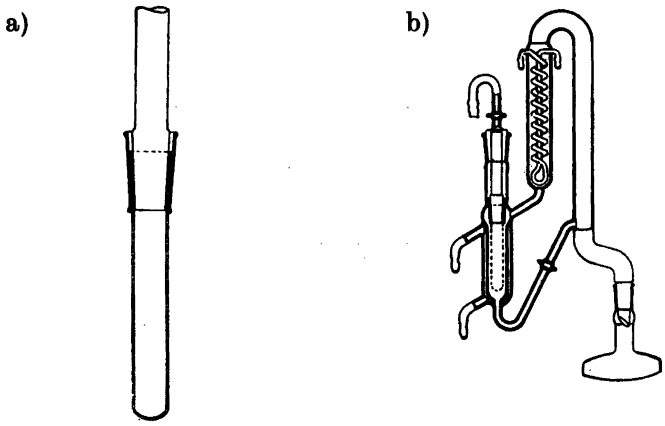
The first indications of the chemical nature of aspartase from propionic acid bacteria were obtained during the different attempts at purifying the enzyme. Its apparent instability was explained by the possible existence of a dissociable low molecular constituent [1]. Later, inhibition studies confirmed this assumption by suggesting one of the alkaline earth metal ions to be essential for the aspartase activity [2].

In this paper attempts have been made to obtain a definite answer to the question of the nature of aspartase as a metallo protein. Therefore the enzyme was subjected to different treatments known to remove dissociable ions, *i. e.* prolonged dialysis, electrodialysis and treatment with ion exchange resins. Using these methods a strong inactivation of the enzyme was obtained. Attempts were made to reactivate the inactivated enzyme by addition of divalent metal ions. A good recovery of the enzyme activity was obtained with a preparation inactivated by a procedure that removed the metal rapidly from the enzyme. The enzyme used in this investigation was obtained from propionic acid bacteria.

EXPERIMENTAL

Enzyme preparation. Propionibacterium petersonii (strain of this laboratory) was the starting material. A crude enzyme solution was prepared exactly as earlier described [1]. The enzyme solution was filtered by aid of Hyflo Super Cel.

Fig. 1. Continuous dialysis apparatus



a) dialysis tube fitted between cone and socket
b) apparatus with the dialysis tube put in.

Activity measurements. The initial velocity of the reaction served as a measure of the enzymatic activity and was obtained exactly as earlier described [1]. The activity is expressed as a percentage of the initial velocity of the untreated enzyme preparation used as control.

The test solution was 0.02 *M* substrate (L-aspartic acid or fumaric acid and ammonia. The acids were neutralized with sodium hydroxide to pH 7.2) in 0.007 *M* tris-(hydroxymethyl)-aminomethane buffer [3] at pH 7.2 in a final volume of 5 ml.

Ammonia was determined according to the method of Conway and O'Malley [4] by distillation into alcoholic boric acid in Conway units and titrated with hydrochloric acid. The hydrogen ion concentration was determined with a glass electrode (Beckman). Protein nitrogen was determined by a micro-Kjeldahl method or, when results comparable with each other only were required, the relative protein concentrations were determined on the basis of the absorption at 280 mμ.

Analysis of metals. A qualitative determination of some metals was performed spectrophotometrically using a Beckman Model DU spetrophotometer with its original flame attachment. Hydrogen-oxygen was used as gas mixture.

Procedure for dialysis. The enzyme solution was subjected to prolonged dialysis against distilled water *in vacuo* using a continuous dialysis apparatus [5].

The apparatus was operated in the following manner: The dialysis tube was attached to the cone of a ground glass joint and the corresponding socket so placed that the dialysis tube was firmly held in the cone and socket. The cone and socket were held by rubber bands.

The enzyme solution (30 ml) was pipetted into the dialysis tube and the tube allowed to hang from the narrowing in the dialysis chamber in the apparatus (Fig. 1 b). Distilled water was placed in the apparatus. The stopper was attached and the apparatus evacuated with a water pump. The distillation flask was heated and the condensed water ran into the dialysing chamber. The displaced water ran through the connecting tube to the distillation flask.

The enzyme activity and protein concentration were determined at regular intervals. The activity was calculated on the same protein content. After the dialysis experiment the distillation flask was analyzed on metals with a flame photometer.

Electrodialysis. The apparatus was of a design similar to that of Albers and Albers [6]. Each compartment had a capacity of approximately 100 ml. The membranes between the compartments were of cellophane. The platinum electrodes were placed close to the membranes. The enzyme solution was placed in the middle compartment. The contents of the middle compartment were stirred to avoid precipitation on the membranes.

Ion-exchange resins. Amberlite IR-120, analytical grade, was washed with water and treated with 5 % hydrochloric acid until the solution remained strongly acid, after which it was washed with distilled water till the water remained nearly neutral (pH ~ 6). The sodium and calcium forms of the resin were prepared by treatment with 5 % sodium and calcium chloride until all the hydrogen ions were displaced, *i. e.* the salt solution remained neutral (pH ~ 6). The resins were dried at 40° C.

The deionization experiments with the different forms of the resin were performed in the following way: 10 ml of the enzyme solution were pipetted into a 100-ml Erlenmayer flask and 1 g of resin was added. When a larger amount of enzyme solution was treated the same ratio between weight of resin and volume of enzyme solution was maintained. The flask was attached to a motor by means of a glass rod through its rubber stopper. The flask was allowed to spin in an oblique position to keep the resin moving continuously in the solution. The decrease in solvent volume was always taken into account in the calculations of activity.

Incubation experiments. A constant amount of enzyme was incubated with varying concentrations of metal ions in 0.007 *M* tris-(hydroxymethyl)-aminomethane buffer at pH 7.2 in a final volume of 4 ml at 20° C for 20 h with 0.03 ml of toluene as antiseptic. The reaction was started by addition of aspartic acid to a final concentration of 0.02 *M* in 5 ml.

Fumarase activity. The fumarase activity of different enzyme preparations were determined by the spectrophotometric method of Racker [7] using a Beckman model DU spectrophotometer. The test solution in a final volume of 5 ml was 0.02 *M* fumarate in 0.007 *M* tris-(hydroxymethyl)-aminomethane buffer at pH 7.2. The reaction was started by addition of the enzyme to a concentration one third of that used in the activity determinations of aspartase. The change in the absorption at 300 mμ and 20° C was recorded at suitable intervals. The control cell contained all components except fumarate.

RESULTS

A preliminary metal analysis was made on the enzyme preparation dialyzed against distilled water (24 h.) using a flame photometer (Beckman). Of the divalent metals only calcium could be detected by this technique. The following calcium bands were identified: 423 mμ, 544 mμ and 623 mμ. No magnesium bands could be detected in any preparation.

In order to elucidate the role of calcium in the activity of aspartase the activating ability of the Ca^{++} ion was tested by incubating the enzyme at pH 7.2 and 20° C in a solution 0.01 *M* in respect to calcium chloride. A slight increase in the activity of the enzyme could be observed (Fig. 2). Accordingly it was decided to investigate the metal dependence of the enzyme activity in detail. The enzyme preparation was therefore subjected to a prolonged dialysis in a continuous dialysis apparatus in order to remove all dissociable ions. The effect of the dialysis was watched by determining the aspartase activity at regular intervals. The decrease of enzyme activity calculated on the same nitrogen content is shown in Fig. 3. The activity decreased continuously until it disappeared completely, after which the solution in the distillation flask was analyzed for metals. Of the divalent metals only calcium could be identified with the flame photometer. Reactivation of the inactivated enzyme was attempted by incubation at pH 7.2 and 20° C in a solution 0.01 *M* in respect to calcium chloride. Only a rather weak reactivation of the enzyme could be observed (Fig. 4). With another preparation no reactivation was noticed. This disagreement between the ability to reactivate the two preparations may have its explanation in some other factors, *e. g.* in the action of proteolytic enzymes decomposing the active protein during the prolonged experiment.

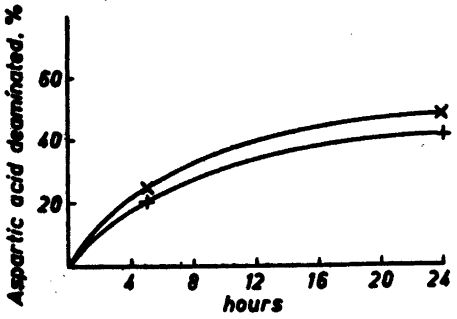


Fig. 2. Activating effect of calcium ion on the deamination of aspartic acid.

× *preparation incubated in 0.01 M calcium chloride*
\+ *control without metal incubation.*

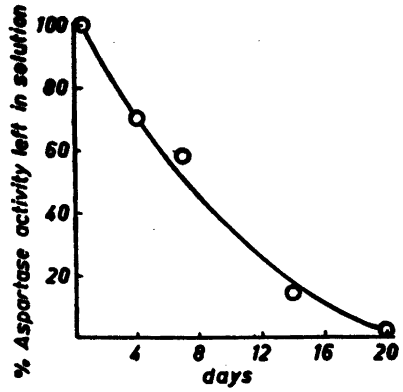


Fig. 3. Effect of prolonged dialysis on the activity of aspartase.

To avoid the disadvantages of prolonged dialysis when factors other than the removal of small ions from the enzyme may also contribute to the loss of enzyme activity, the enzyme was subjected to electrodialysis.

The experiments were carried out with a current of 6—8 mA. In the first experiment the enzyme lost its activity completely within one hour. During this time the pH of the solution decreased to 3.2 and a strong precipitation of

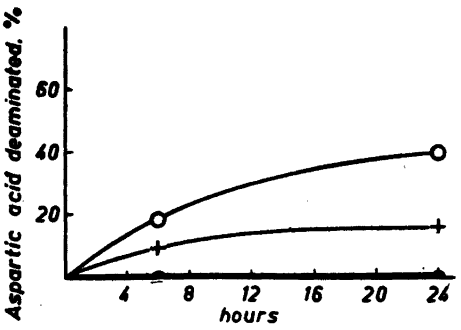


Fig. 4. Reactivating effect of calcium ion on aspartase inactivated by prolonged dialysis

\+ *dialyzed enzyme incubated in 0.01 M calcium chloride*
● *dialyzed enzyme without metal incubation*
○ *control with undialyzed enzyme.*

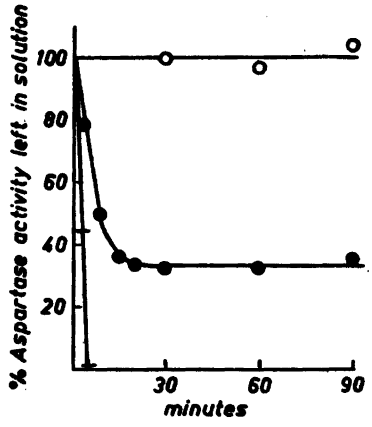


Fig. 5. Inactivating effect of resin treatment on the activity of aspartase

○ *treatment with Amberlite IR-120 (Ca)*
● *treatment with Amberlite IR-120 (Na)*
\+ *treatment with Amberlite IR-120 (H).*

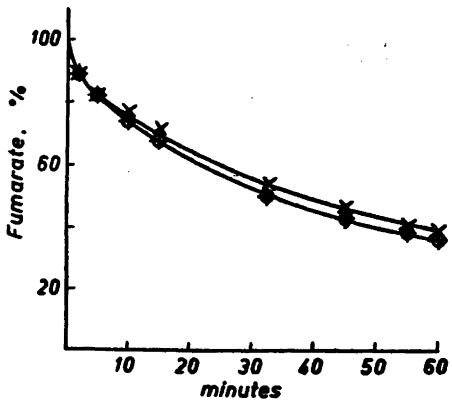


Fig. 6. Effect of resin treatment on the activity of fumarase

\+ *treatment with Amberlite IR-120 (Na)*
× *resin treated enzyme incubated in calcium chloride*
O *control without resin treatment.*

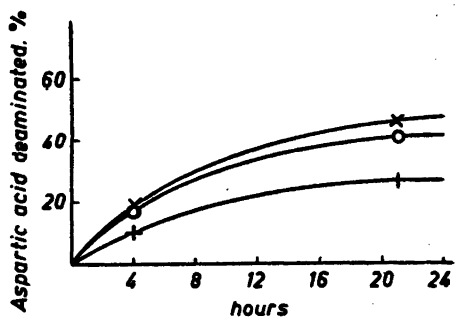


Fig. 7. Effect of resin treatment on the activity of aspartase

\+ *treatment with Amberlite IR-120 (Na)*
× *resin treated enzyme incubated in calcium chloride*
O *control without resin treatment.*

protein occurred. Another experiment was carried out where the pH of the solution was kept constant by addition of alkali. After four hours the preparation had lost its activity completely. In spite of a thorough stirring of the solution during the experiment a strong precipitation of protein on the membranes could not be prevented. For this reason it is difficult to decide if the inactivation of the preparation was due to the fact that the active metal was removed, or to a denaturation of the protein on the membranes. These experiments showed, however, that the preparation contains large anions not able to penetrate the membranes and small cations readily passing through. In both these experiments the solutions in the cathode compartments were analyzed for metals. The Ca bands mentioned above were again detected. When incubated with calcium chloride no reactivation was obtained with these enzyme preparations.

The ability of the cation exchange resin, Amberlite IR-120, to affect the activity of the enzyme was tested. The effect of the hydrogen sodium, and calcium forms of the resin on the enzyme is shown in Fig. 5. The comparison of the effect of the different forms of the resin shows that the Ca form had no inactivation power, whereas the sodium and hydrogen forms strongly inactivated the enzyme. With the sodium and calcium forms no change in the pH could be demonstrated. However, on using the hydrogen form of the resin, the pH of the solution fell rapidly to about 3. (For the activity test the pH was adjusted to 7 by addition of a small amount of 0.1 *N* sodium hydroxide.)

Since aspartase catalyzes the reaction equilibrium

$$\text{Aspartic acid} \rightleftharpoons \text{Fumaric acid} + \text{Ammonia}$$

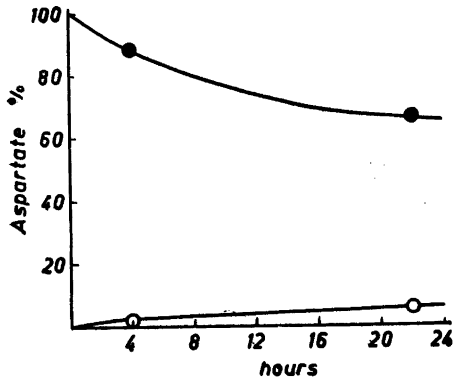


Fig. 8. Synthetic and deaminating effect of aspartase
○ *synthesis*
● *deamination.*

the observed metal dependence of the deamination activity may, of course be due to an activation of aspartase itself, but according to the reaction of equilibrium, any procedure able to decrease the effective concentration of fumaric acid should tend to drive the reaction to the right and so increase deamination at the expense of synthesis. In this case fumaric acid can be removed from the system either by the action of fumarase, converting it to malic acid or, in the presence of free divalent metal ions, by the formation of complexes between the metal and the acid, so decreasing the concentration of the effective acid.

The fumarase activity was determined after treating the enzyme preparation for 50 minutes with the sodium form of the resin as well as after incubation of such a preparation with calcium chloride for 20 h. at 20° C (Fig. 6). Activity determinations of aspartase were made simultaneously for the same preparations (Fig. 7). It can easily be seen that no change in fumarase activity was obtained, the activity of aspartase, on the contrary, being decreased by the resin treatment by about 50 % and completely restored by incubation with calcium chloride, treatments without effect on the activity of fumarase, which is not a metal protein [8].

From the experiments it can easily be seen that the fumarase activity of the preparations is so high that the fumarase equilibrium between fumaric and malic acid should always be established in experiments on aspartase, particularly as the activities in Fig. 6 are obtained at 20° C only and with enzyme concentrations one third of those used for the activity determinations of aspartase at 37° C. (Fig. 7). Hence it is quite evident that the curves of deamination and synthesis of aspartic acid by aspartase fail to agree with each other mainly because of the action of fumarase in converting about 80 % of the fumaric acid in the synthesis experiment to malic acid, before any synthesis of aspartic acid has taken place; moreover the malic acid should act as an inhibitor of aspartase. The difference in the deamination and synthetic activities of the same aspartase preparation can be seen in Fig. 8. The experiments are performed in the same molar concentration of substrates and are therefore comparable with each other.

To ascertain the extent to which the increase in the deamination activity may be due to a shift of the equilibrium toward deamination because of a

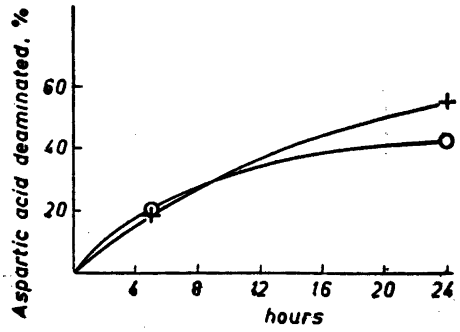


Fig. 9. Effect of excess calcium ion on deamination of aspartic acid
+ deamination in 0.1 M calcium chloride
O control without addition of calcium chloride.

binding of effective fumaric acid in a complex with the activating metal ion in question, activity determinations were made in test solutions containing calcium ion in excess (Fig. 9). It is evident that a shift toward deamination can be obtained in this way, although it can be seen from the curves that no activation occurred. A similar shift toward deamination has earlier been shown by Jacobsohn and Pereira to occur with Mg^{++} ion [9]. It appears that the activation cannot be explained in this way. Accordingly the main activation is due to the activation of aspartase itself.

Table 1. Reactivation of partially inactivated aspartase by incubating the enzyme with different metal ions at 20° C and pH 7.2 for 20 hours.

Treatment of enzyme before incubaton	Incubation		Activity as percentage of control
	Metal salt	Final metal concentration Mole / 1.	
Control (untreated)	—	—	100
Amberlite IR-120 (Na)	—	—	43
—»—	$CaCl_2$	0.01	103
—»—	$CaCl_2$	0.001	102
—»—	$CaCl_2$	0.0001	90
—»—	$CaCl_2$	0.00001	77
—»—	$CaCl_2$	0.000001	44
—»—	$MnCl_2$	0.01	104
—»—	$MnCl_2$	0.001	101
—»—	$MnCl_2$	0.0001	96
—»—	$MnCl_2$	0.00001	88
—»—	$MnCl_2$	0.000001	45
—»—	$BaCl_2$	0.01	80
—»—	$BaCl_2$	0.001	51
—»—	$SrCl_2$	0.01	100
—»—	$SrCl_2$	0.001	51
—»—	$MgCl_2$	0.01	100
—»—	$MgCl_2$	0.001	58
—»—	$CoCl_2$	0.001	54
—»—	$NiCl_2$	0.001	41
—»—	$FeCl_2$	0.001	19
—»—	$ZnCl_2$	0.001	42
—»—	$PbCl_2$	0.001	51

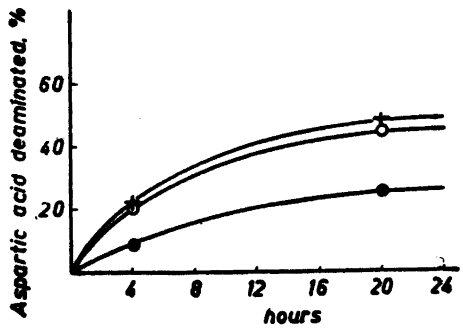


Fig. 10. Reactivation of resin treated aspartase (Amberlite IR-120 (H))
\+ *neutralized with calcium carbonate*
● *neutralized with sodium hydroxide*
O *control without resin treatment.*

To ascertain to what extent the activating power of calcium ions is shared by other divalent metal ions with known biological activity, the enzyme solution was deionized with the sodium form of IR-120 (50 min.) and incubated with different metal ions. The final volume of the test solution was 5 ml. The reactivating power of the different metal salts can be seen in Table 1.

It can be seen from Table 1 that Ca^{++} and Mn^{++} showed the strongest reactivating power even at low concentrations. It is therefore evident that Ca^{++} is the natural activator of the aspartase of propionic bacteria. Reactivation was also produced by such metal ions as Mg^{++}, Ba^{++} and Sr^{++}, but at higher concentrations only. Metal ions like Co^{++} and Pb^{++}, earlier found to be inhibitors to the enzyme [2], showed a slight reactivating power at low concentration. No activation or inactivation was produced at low concentrations by the metal inhibitors [2] Ni^{++}, Fe^{++} or Zn^{++}.

In order to find out if the protein as such is inactive, attempts were made to obtain a complete inactivation but still keeping the reactivating ability unchanged. The enzyme was therefore rapidly inactivated with the hydrogen form of the resin and neutralized with solid calcium carbonate to about pH 6.5. The corresponding control was neutralized with sodium hydroxide to the same pH. A complete reactivation was obtained using this technique if the pH of the solution was not allowed to fall below 4 (Fig. 10) during the resin treatment. If, however, the resin treatment was prolonged until the pH of the

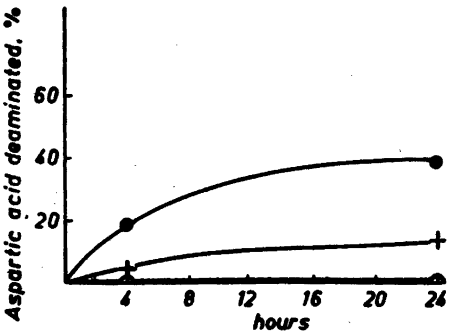


Fig. 11. Reactivation of resin treated aspartase (Amberlite IR-120 (H))
\+ *neutralized with calcium carbonate*
O *neutralized with sodium hydroxide*
● *control without resin treatment.*

solution was about 3, no activity remained in the solution neutralized with sodium hydroxide, but when neutralized with solid calcium carbonate a slight reactivation was obtained (Fig. 11).

DISCUSSION

In a recent paper it was concluded from inhibition data that aspartase is a metal protein, one of the alkaline earth metals being essential for its activity [2]. The present findings provide convincing evidence for this conclusion.

From the reactivation experiments it can be seen that aspartase has a low metal ion specificity, although the individual ions have different reactivating efficiency. In this respect aspartase shows similarities to peptidases [10], carboxylases [11] and phosphatases [12]. The interchangeability of the activating metal ions can be explained by considering the ways in which different metal ions form complexes. In biological systems the fixed pH (usually about 7) limits the types of complexes which various metal ions can form [13]. Thus it is known that metal ions such as the cupric ion will be bound in nitrogen complexes rather than in oxygen complexes; hence they will be bound to different groups in an enzyme than will cations such as Mg^{++} and Mn^{++}. Between the ions which behave like Cu^{++}, Ag^{+}, and Pb^{++}, and those similar to Mg^{++}, such as Ca^{++} and Mn^{++}, there will be a group which is intermediate in character, forming mixtures of oxygen and nitrogen compounds *i. e.* Co^{++}, Zu^{++} and Cd^{++}. On adding an enzyme to a solution of a metal ion a complex of any of these three types will form depending on the nature of the metal. From earlier inhibition studies it was realized that the metal ion which activated aspartase formed strong oxygen complexes, a fact in good agreement with the findings in this paper that the oxygen complex-forming metal ions have the strongest activating power.

Recently Trudinger [14] found that aspartase activity of toluene-treated cells of *Proteus vulgaris* sp. was stimulated by such metal ions as Mg^{++}, Mn^{++} and Co^{++} only at substrate concentrations below 0.01 *M*. Ca^{++} and Ba^{++} had no activating effect, a finding contrary to that in this paper concerning the enzyme from propionic acid bacteria. Moreover, it is somewhat surprising that the activation is limited to low substrate concentrations only, so that, for instance, at a substrate concentration of about 0.02 *M* no activation could be observed. All the experiments in this paper were performed at 0.02 *M* substrate concentration. It is true, however, that hardly any activation of the enzyme was obtained until it was partly inactivated by removing the metal, after which a complete reactivation of the original activity was obtained by incubation with divalent metals.

A difference was also observed by Trudinger between the activation of different bacteria, *e. g. Aerobacter aerogenes* exhibited a marked metal ion dependence while *Escherichia coli*, on the contrary, seemed to be less dependent on metal ions.

From the experiments in this paper it can be concluded that at least 60 % of the aspartase activity depends on activation by metals. Whether the protein component as such is completely inactive cannot be decided on the basis of these experiments. However, the possibility should not be entirely over-

looked that the metal may be replaced by such groups as cationic $H_2N^+=C(NH)—NH—$ or $H_3N^+—$groups from arginine or lysine side-chains.

These positively charged loci with their ability to form hydrogen bonds could function in the same way as Me^{++}. In such a case one might expect to find non-specific anion inhibitors for this enzyme, even though it is not metal-activated. However, the inhibition studies on aspartase from propionic acid bacteria are completely explained as an inhibition of the metal-activated enzyme, if it is assumed that only 60 % of it is metal-activated.

I wish to express my sincere gratitude to Professor A. I. Virtanen for continued interest and valuable discussions in the work on aspartase.

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Chromatographic Studies on Spruce Bark Tannin Extract

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Solid technical spruce bark tannin extract as well as extracts from fresh bark of Norway spruce, *Picea abies* (L) Karst., have been studied by two-dimensional chromatography. A large number of phenolic and fluorescent components were detected along with some aldehydes or ketones. A few components of the technical extract could not be found in fresh bark, and *vice versa*. The acetone extract of the technical extract was fractionated by Craig distribution between ethyl acetate and water (51 fractions). The intermediate fractions, which had the least complicated composition, were further investigated by partition chromatography. Some phenolic components having a strong bluish-violet fluorescence were greatly concentrated but could not be isolated in a pure state. One component of the technical extract, which is probably an artefact containing sulphur, showed a tendency to crystallise but was very unstable when isolated. The ultraviolet light absorption of three of the substances was investigated after elution from the chromatograms.

In spite of the great technical importance of the vegetable tannins, very little is at present known about their chemical structure. The main reason for this is the extremely complex composition of vegetable tannin extracts, as has been shown in recent papers by White and co-workers [1-6]. These authors, using paper chromatography, were able to demonstrate that all vegetable tannin extracts contain a very large number of components. Fractionation of these components by extraction and precipitation methods could be easily followed by paper chromatography.

In recent years, a few crystalline tannins of known composition have been isolated from divi-divi and from myrobalans by Schmidt and co-workers [7]. These substances belong to the so-called hydrolysable tannins. From the condensed tannins, no pure compound with tanning properties has been isolated so far, all publications about their chemical structure being entirely speculative. With the information now available concerning the complexity of these tannins, it must be regarded as an almost hopeless task to try to isolate any pure compounds from them by classical methods. The application of modern

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methods of fractionation, such as chromatography and counter-current extraction, might be expected to give better results.

The bark of Norway spruce, *Picea abies* (L) Karst., is the richest potential source of vegetable tannins in Scandinavia. At present it is utilised for tanning to a very small extent. Previous investigations on spruce bark extract have mainly dealt with its practical value as a tannin extract, and the changes in tanning properties on fractionation [8–14]. In lectures, Grassmann has twice reported the isolation of crystalline substances from spruce bark [15–16], but no further publications have appeared so far.

The present investigation was mainly carried out on solid technical spruce bark extract from AB Tannin, Västervik, Sweden. For comparison, a few extracts of fresh spruce bark were also made in the laboratory. The investigation of the technical spruce bark extract was limited to the acetone-soluble part of it, varying between 25 and 40 % of different samples of solid extract.

Küntzel and Melzer [11] and, later, Haglund [12] have carried out fractionations of aqueous spruce bark extract by precipitation with ethanol. According to Haglund [12], the fraction which is not precipitated in 80 % ethanolic solution contains the greater part of the substances which are valuable for tanning. The ethanol-insoluble fraction contains large quantities of salts and yields a poor leather on tanning. The properties of our acetone extract were found to correspond roughly to those of Haglund's soluble fraction. The acetone-insoluble residue, containing large amounts of salts (ashes 5.1 %), gives very diffuse spots on the paper chromatogram and must be regarded to be less suitable for attempts to isolate pure substances.

PAPER CHROMATOGRAPHY

For analysis of the different fractions of the spruce bark extract, we used two-dimensional paper chromatography. The solvents were those recommended by White and co-workers for other tannins [3], namely 0.1 % acetic acid in water, saturated with *tert.*-amyl alcohol in one direction and *sec.*-butanol (water-saturated) in the other. To make the spots visible, we employed the following methods: 1) Observation of the paper in ultraviolet light before and after treatment with ammonia vapour. 2) Spraying the paper with *bis*-diazotised benzidine solution [17]. This method has previously been used successfully by one of us for identifying phenolic pine heartwood constituents [18]. 3) Spraying with ammoniacal silver nitrate and 4) Spraying with a solution of 2,4-dinitrophenylhydrazine hydrochloride [19].

Methods 1) and 2) are the most important ones, method 2) being specific to phenols (especially polyphenols) and aromatic amines, but method 1) generally being more sensitive. Both methods gives spots of varying colours, which facilitates the identification. The fluorescent spots are generally bluish violet, but in some cases yellow or green. The colours produced by diazotised benzidine are red, yellow or brown. Method 3) is applicable to reducing substances in general, many of which also react with reagent 2). The reducing sugars, which do not react with any of the other reagents, are also detected by method 3). Reagent 4) was used only in special cases for the detection of aldehydes and ketones.

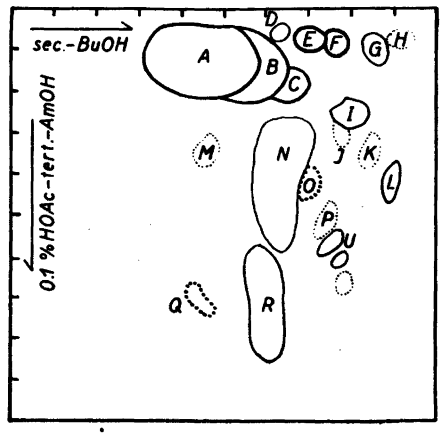


Fig. 1. Two-dimensional paper chromatogram of the acetone extract of solid technical spruce bark extract before fractionation. The starting point is at the upper left corner. Paper: Whatman No 2. Full contours: fluorescent areas. Dotted contours: non-fluorescent areas, detectable by colour reaction with diazotised benzidine. The letters refer to Table 1.

In Fig. 1, a two-dimensional chromatogram of the entire acetone-soluble fraction, before further fractionation, is demonstrated. The picture has been slightly simplified by omitting some very weak and diffuse spots. The chromatogram is entirely dominated by the three spots A, B, and C, giving a strong bluish-violet fluorescence and strong red or brown colours with diazotised benzidine. Although there are many other fluorescent spots on the chromatogram, these three substances must be mainly responsible for the strong bluish-

Table 1. Colour reactions of the strongest spots on the chromatograms of spruce bark extract. This table refers to the spots visible on Fig. 1 and later figures.

Spot	Fluorescence	Fluorescence in NH_3 vapour	Colour reaction with diazotised benzidine
A	bluish-violet	bluish-green	brown
B	» »	bluish-white	red
C	» »	bluish-violet	red
D	yellow	yellow	red
E	blue	green	red
F	blue	blue	red
G	—	green	red
H	—	—	red
I	—	blue	red
J	—	—	red
K	—	—	red
L	—	blue	—
M	—	—	brownish-yellow
N	—	blue	—
O	—	—	brownish-yellow
P	—	—	red
Q	—	—	red
R	—	blue	—
S	—	—	red
T	—	—	brownish-yellow
U	blue	green	red
V	—	—	brownish-yellow
W	—	—	brownish-yellow

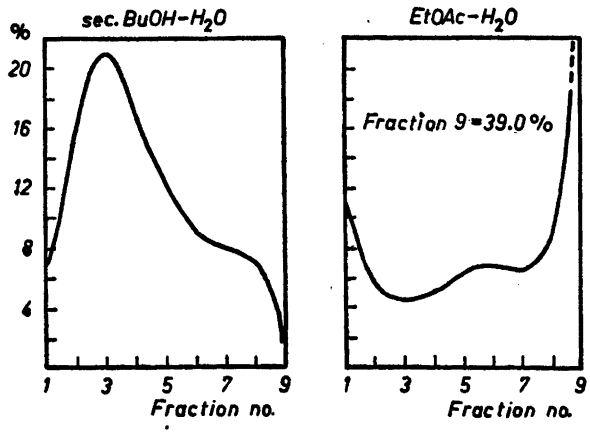


Fig. 2. Craig distribution diagrams (nine fractions) for the acetone extract of solid technical spruce bark extract between sec.*-butanol and water (left) and between ethyl acetate and water (right).*

violet fluorescence of spruce bark. Many years ago, Gerngross made an attempt to isolate the fluorescent component from an ether extract of spruce bark [20–21]. He called it "violettin", believing it to be a single substance, but it is evident that his preparation must have been a very complex mixture.

The colour reactions of the strongest spots on the chromatograms are listed in Table 1. The R_F values are not mentioned in the table but can be approximately estimated from Fig. 1. It is evident that most of the spots give a fluorescence in ultraviolet light. With few exceptions, these spots also give a colour reaction with diazotised benzidine. In some cases, the failure of a colour reaction may be due to a too low concentration of the substance, since the fluorescence is more sensitive than the colour reaction. Finally, there are some non-fluorescent spots giving a colour reaction. A few of these spots give a strong brownish-yellow colour (M, O), while others give a red colour with diazotised benzidine (H, J *etc.*).

COUNTER-CURRENT DISTRIBUTION

For further fractionation of the extract, we used the Craig counter-current distribution technique, which has been previously employed by White and co-workers for fractionation of other tannin extracts [3,5]. Fig. 2 shows the weight distribution curves from two pilot experiments with ethyl acetate-water and *sec.*-butanol-water as the solvent pairs. The number of fractions was nine in both cases. Fig. 2 clearly shows that ethyl acetate-water sharply divides spruce bark extract into an ethyl acetate-soluble and a water-soluble fraction with relatively small intermediate fractions. On distribution between *sec.*-butanol and water, on the contrary, the yield is mainly concentrated to the intermediate fractions. Reducing sugars (detectable with aniline hydrogen phtalate) were found only in fractions 7—9 from the ethyl acetate-water distribution, but in

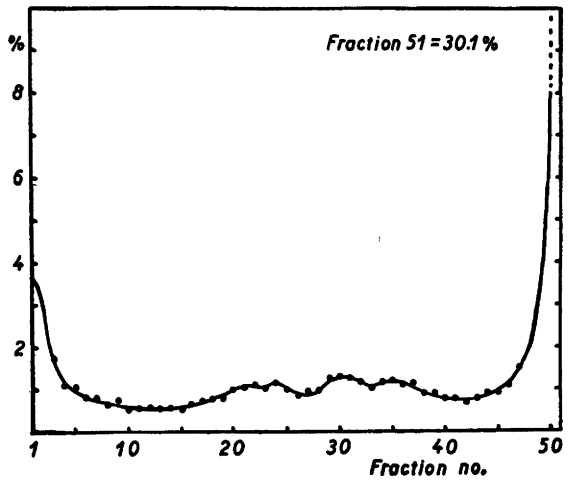


Fig. 3. Craig distribution diagram for the same material as in Fig. 2 between ethyl acetate and water (51 fractions).

the *sec.*-butanol experiment, fractions 3—9 gave a positive sugar reaction. A paper chromatographic analysis of the fractions also showed that the ethyl acetate fractionation is far more promising with regard to the separation of the phenolic spruce bark constituents.

A larger quantity (75 g) of the acetone extract was distributed between ethyl acetate and water in 51 fractions. Fig. 3 shows the weight distribution curve from this experiment. As can be seen from the curve, the fraction most soluble in water (No. 51) contains 30 % of the starting material and fractions 47—51 together more than 45 %. Another maximum is represented by the fractions most soluble in ethyl acetate. (Fractions 1—5 contain more than 10 % together.) In the intermediate fractions, the material is more evenly distributed, but some less pronounced maxima and minima can be observed. None of these fractions contains more than 1.5 % of the starting material.

The chromatograms of the fractions from the counter-current distributions indicate that a rather good fractionation of the material has been achieved. The total number of spots visible on the different chromatograms exceeds that which can be observed on Fig. 1. In Fig. 4, a number of typical chromatograms from some of the fractions are represented. As regards the minor components, one must not neglect the possibility of new substances being formed by oxidation or other reactions during the distribution, which lasted for more than one month. Although a fairly good enrichment of the components has been achieved in certain fractions, we are, however, at this stage still a long way from the isolation of any pure compounds. Each one of the main components occurs in at least ten different fractions, and each fraction still contains several components visible on the chromatograms.

In Fig. 4, the chromatograms from six Craig fractions of different types are represented. The most ethyl acetate-soluble fraction (No. 1) contains a large

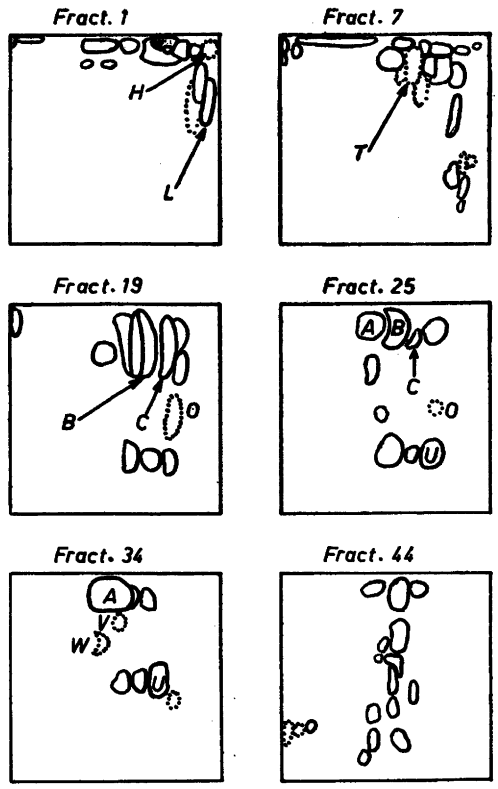


Fig. 4. Two-dimensional chromatograms of six fractions from the Craig distribution. The letters refer to Table 1.

number of substances. Most spots on the chromatogram are crowded along its upper edge. Some of the spots have a yellow or brownish fluorescence, in contrast to the dominating blue or violet colour. One of the strongest spots on the chromatogram is L, which always has an oblong shape and is detected by its strong blue fluorescence in ammonia vapour. This spot has been found in fractions 1—6.

The chromatogram of fraction 7 is rich in spots, many of which are weak and diffuse. The dominating spot is T, which is non-fluorescent. On the chromatogram of the whole acetone extract, it is very likely covered by the strong spots A and B.

The intermediate fractions (about Nos. 13—40) seem to be of less complicated composition than the first and last ones. All the strongest spots on the chromatogram reach their maximum intensity within this interval, and the number of minor components is relatively small. These fractions must be judged to be the most suitable starting material for attempts to isolate any pure substances. In Fig. 4, three fractions from this group are represented.

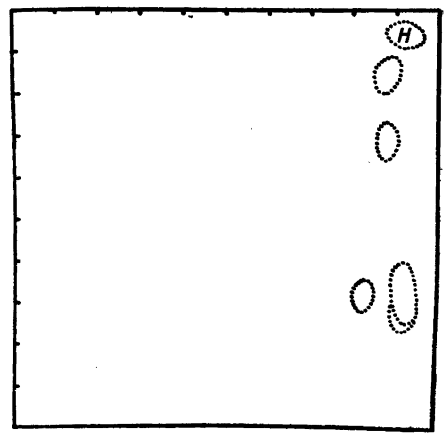


Fig. 5. Position of spots giving a colour reaction with 2,4-dinitrophenylhydrazine, which are found on the chromatograms of the Craig fractions.

About fraction 19, the spots C and O (which is not very strong on Fig. 1) reach their maxima. Fraction 25 represents the maximum of B, while C and O have almost vanished and A is still weak. Two rather strong spots have developed below O. In fraction 34, A dominates together with U, a spot giving a green fluorescence in ammonia vapour. Two small non-fluorescent spots (V, W), giving a similar colour reaction as O, have developed below A.

Fraction 44 is an example of the last fractions, giving a chromatogram containing a large number of relatively weak spots, most of which are gathered along a straight line across the middle of the chromatogram. These fractions must be less suited for isolation experiments.

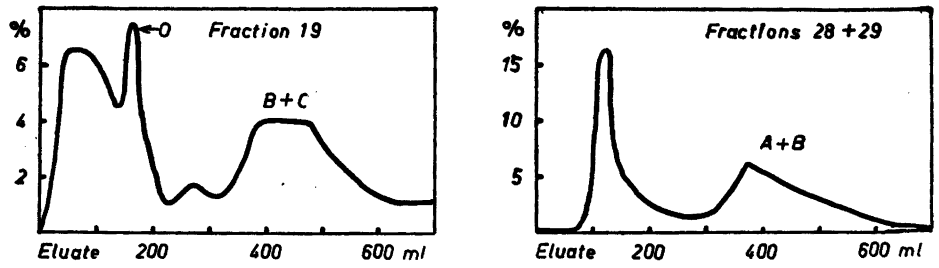
The very last fractions (48—51) give hardly any spots which are visible by fluorescence or benzidine colour reaction. Instead, a very strong reaction of reducing sugars is obtained on spraying with ammoniacal silver nitrate or aniline hydrogen phtalate. At present, nothing can be said about the composition of these fractions, except that they contain carbohydrates. In fraction 51, the content of "tannins", determined by the shake method, was found to be 11.6 %. The corresponding figure for the whole acetone extract was 48 %. Thus, fraction 51 contains only very little of the material which is determined as "tannins" by the official analysis. Of course, the substances in fraction 51 may still play an important part in tanning, although they do not act as tannins when separated from the other constituents of the extract.

At present, it is impossible to distinguish between "tannin" and "non-tannin" spots on the chromatograms.

As mentioned above, 2,4-dinitrophenylhydrazine was also used as a spraying reagent for the detection of aldehydes and ketones. Chromatograms of fractions 1—14 were treated with this reagent, and Fig. 5 shows all the "carbonyl" spots found on these chromatograms. The spots which are situated high up on the paper are found in the first fractions, the lower spots coming out in the higher fractions. All these spots are non-fluorescent. One of them, developing in fractions 1—5, seems to be identical with spot H (see Fig. 1).

PARTITION CHROMATOGRAPHY

As mentioned above, the Craig distribution did not lead to isolation of any pure substances. In order to separate the components of fractions 14—40, partition chromatography on cellulose columns was therefore tried. Water-acetone (4:1), containing 0.1 % acetic acid, was used as a solvent. The fractions of the eluate were evaporated to dryness, weighed and analysed by paper chromatography. Fig. 6 shows a weight distribution curve of the eluate from fraction 19. (*Cf.* Fig. 4). There are three major weight maxima, the second of which corresponds to substance O and the third to B+C and some minor components. The first one contains the fluorescent compounds coming out below O on the chromatogram, but since the intensity of these spots is much lower than that of O, B or C, one must assume that the bulk of the first weight maximum must be due to some substance which cannot be detected on the chromatogram by the methods used here.



Figs. 6 and 7. Weight distribution in the eluate from the cellulose column separations of Craig fraction 19 (Fig. 6) and fractions 28 + 29 (Fig. 7). Each fraction of eluate = 25 ml.

Fig. 7 represents the weight distribution curve from the separation of fractions 28+29 on a cellulose column. The first very sharp weight maximum did not give any strong spots on the chromatogram. The second one is mainly due to compounds A and B but also to some minor components.

The other column separations gave results similar to those demonstrated in Figs. 6 and 7. In no case did we obtain a fraction containing only one of the fluorescent compounds, although they had been greatly concentrated compared with the starting material, and none of these fractions showed a tendency to crystallise. Some fractions, however, seemed to consist mainly of the non-fluorescent compound O (see Table 1 and Fig. 6), and a light brownish crystalline material was obtained on evaporation to dryness. When attempting to recrystallise it, however, brown amorphous products were formed. A qualitative analysis of the crude product gave a positive reaction for sulphur. Substance O may be assumed to be a phenolic sulphonic acid, which has been formed during the weak sulphitation of spruce bark which is carried out when preparing the technical extract. There are some other non-fluorescent spots found on the chromatograms giving the same brownish-yellow colour with diazotised benzidine. None of these spots were found on the chromatogram of fresh spruce bark extract (see below).

It is remarkable that chebulinic acid, chebulagic acid and corilagin (kindly put at our disposal by Prof. O. Th. Schmidt, Heidelberg) give a colour reaction which is similar to that given by the substances just mentioned. These three substances have been isolated from divi-divi and myrobalans [7].

From the fractionation experiments just described, one can draw the conclusion that the compounds corresponding to the strongest spots on the chromatogram (A, B, C, and O) constitute a considerable part of the intermediate fractions, and thus their isolation should not be impossible. On the other hand, these same fractions contain a large percentage of substances which cannot be made visible on the chromatogram by the methods used here. At present, nothing can be said about the chemical nature of these substances, except that some of them may be glycosides. On boiling with dilute hydrochloric acid, reducing sugars could be detected in the solution. These substances always had higher R_F values on the cellulose column than the phenolic substances.

EXTRACTS OF FRESH SPRUCE BARK

The experiments hitherto described were all carried out with commercial spruce bark extract, which is available on the market in large quantities. This extract is prepared by treating the bark with hot water containing a little sodium sulphite or bisulphite, which renders part of the so-called phlobaphenes soluble, thus increasing the yield of extract (see below). From the tanner's point of view, the value of the sulphitation is dubious [11]. Thus, the technical extract may contain sulphur compounds which are not present in the bark, and, in addition, the components of the bark may have been changed by oxidation before the extraction. Extracts of bark, which had just been removed from a living spruce, were therefore prepared. The greater part of the outermost layer of the bark was removed before extraction. The yields are listed in Table 2.

Table 2. Yields of extract of fresh spruce bark with different solvents.

Solvent	Yield, %
Acetone	11
Water	17
Water with Na_2SO_3 + $NaHSO_3$ (0.5 % of the bark of each)	33

A chromatogram of the acetone extract is shown in Fig. 8. The chromatogram of the water extract is very similar, and the higher yield of the latter extract is probably due to an increased extraction of carbohydrates. The sulphite extract, on the other hand, contains some phenolic components which are found neither in the other extracts nor in the technical extract.

If Fig. 1 and Fig. 8 are compared, it is evident that the three strongest fluorescent spots, A, B, and C, are present on both chromatograms. Thus, to study these substances, it is possible to start from the technical extract as well as from fresh spruce bark. The most important difference between the two

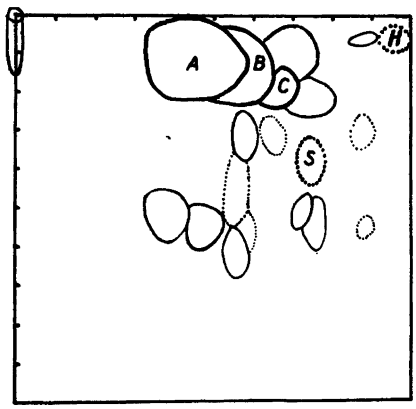


Fig. 8. Two-dimensional chromatogram of the acetone extract of fresh spruce bark. The letters refer to Table 1.

extracts is that compound O and the other non-fluorescent substances with a brownish-yellow colour reaction are totally absent in fresh spruce bark. On the contrary, fresh spruce bark contains, as a main constituent, a non-fluorescent substance giving a strong reddish colour with the benzidine reagent (S), which is absent in the technical extract. On the whole, the total number of minor constituents seems to be larger in the technical extract.

All attempts to obtain compound O in the laboratory by extracting spruce bark with water or sulphite solutions of varying strength failed. This compound must have been formed under the special conditions prevailing during the technical extraction.

ULTRAVIOLET LIGHT ABSORPTION OF SOME OF THE COMPOUNDS

The ultraviolet light absorption of three of the substances (A, O, S) in ethanol solution was determined by elution of the spots from a number of paper chromatograms. All absorption curves had one maximum and one minimum, the wavelengths of which are listed in Table 3.

Table 3. Maxima and minima of ultraviolet light absorption.

Substance	Maximum	Minimum
A	251 mμ	240 mμ
O	262 mμ	239 mμ
S	272 mμ	256 mμ

EXPERIMENTAL

The acetone extractions of solid technical extract were carried out in a large percolator for 24 h. The extract was concentrated in a nitrogen atmosphere and finally dried in a vacuum drying oven at 40°. The extractions of fresh bark were carried out in a Sox hlet apparatus.

The Craig distribution was carried out as follows: Ordinary bottles, provided with sealed-in outlet tubes, were used as separating vessels. Each bottle, in the beginning,

contained 500 ml of water-saturated ethyl acetate. In the first bottle, 500 ml of water, saturated with ethyl acetate, and 75 g of the acetone extract (dry weight 70 g) were also introduced. The bottle was shaken, and the two phases allowed to settle. The aqueous phase was then drawn off through the bottom tube into bottle No. 2, and fresh aqueous phase (500 ml) was introduced into bottle No. 1. Both bottles were then shaken, whereupon the bottom phase of bottle No. 2 was transferred to bottle No. 3, that of bottle No. 1 to bottle No. 2, and fresh water added to bottle No. 1. The fractionation was carried on in this way until the material was divided into 51 fractions. Sometimes, the liquid phases formed emulsions which had to be broken up by centrifugation. Both phases of each fraction were concentrated together by distillation in a nitrogen atmosphere and finally dried in a vacuum. The residues were weighed and the residual moisture content determined by drying a small sample at 105°.

Glass tubes (size 60 × 3.5 cm), packed with cellulose powder (200 mesh), which had previously been boiled with 5 % nitric acid and thoroughly washed with water and acetone, were used for the chromatographic separations. The solvent consisted of 0.1 % acetic acid in water (4 vols.) and acetone (1 vol.). About 0.5 g of material, dissolved in a small volume of solvent, was added to the top of the column by a pipette. The top was covered by a little more cellulose and the solvent allowed to flow through the column. The movement of the fluorescent zones could be observed by illuminating with a quartz lamp. The eluate was divided into 10 to 25 ml fractions, which were evaporated to dryness, weighed and analysed by paper chromatography.

For two-dimensional chromatography, Whatman No. 2 papers (size 23 × 23 cm) were used. The solvents were those described by White and co-workers [3].

Solvent 1: 0.1 % acetic acid in water, saturated with *tert.*-amyl alcohol.

Solvent 2: Sec.-butanol, saturated with water.

The chromatograms were run with Solvent 1 for 8 h, dried and then run at right angles with Solvent 2 for 2 h.

Spraying reagents

Diazotised benzidine [17, 18]. Benzidine (5 g) was stirred with conc. hydrochloric acid (14 ml), and the suspension dissolved in water (980 ml). Just before spraying, a 10 % sodium nitrite solution was added to this solution dropwise under stirring until the dark colour first formed turned to light yellow. The mixed solution is not stable. After spraying with this reagent, the papers must be immediately washed with water to avoid a dark background coloration.

Ammoniacal silver nitrate [3]. 5 % aqueous silver nitrate solution (20 ml) was mixed with sufficient 10 % ammonia solution to obtain a clear solution, and the volume then made up to 100 ml. After spraying, the paper was dried at 105° for 10 min. The excess reagent may be removed by washing with sodium thiosulphate solution.

2,4-dinitrophenylhydrazine hydrochloride [19]. Dinitrophenylhydrazine (1 g) was dissolved in conc. hydrochloric acid (300 ml), and the solution diluted to 1 l with water. This reagent forms yellow spots with aldehydes and ketones. The spots must be marked with a pencil immediately after spraying, since they soon become less distinct owing to background coloration.

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Hippurylcholine

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Hippurylcholine, the hippuric acid ester of choline, has been synthesized and its chemical, biochemical and pharmacological properties described.

Hippurylcholine is not identical with the "F component" present in ox spleen. This component may be a choline ester of an aromatic acid derivative containing glycine.

During investigations in this laboratory concerning the natural occurrence of choline esters other than acetylcholine in animal organisms, *hippurylcholine* or a derivative thereof was thought to be present in ox spleen and identical with the unidentified ester ("F component") reported by Banister, Whittaker and Wijesundera [1]. Hippurylcholine was therefore synthesized and tested in the chromatographic analysis of the isolated bases from the spleen. It was demonstrated, however, that the new ester is neither identical with the "F component", nor present in the material studied.

Hippurylcholine, the hippuric acid ester of choline, has not hitherto been described in the literature. Its synthesis together with some of its chemical, biochemical and pharmacological properties are reported briefly in the present paper.

Chemistry. Hippurylcholine chloride (HiCh), $C_6H_5CONHCH_2CO\text{-}OCH_2CH_2N^+(CH_3)_3Cl^-$, was prepared by methylating β-chloroethyl hippurate, $C_6H_5CONHCH_2CO\text{-}OCH_2CH_2Cl$. The latter compound was synthesized from hippuric acid and 2-chloro-ethanol. The methods used were similar to those described by von Euler *et al.* [2].

HiCh crystallizes with one molecule of water in white non-hygroscopic prisms, m. p. 91°, very soluble in water and ethanol, insoluble in ether, ethylacetate, chloroform, benzene, acetone. The rate of spontaneous hydrolysis in distilled water is similar to that of acetylcholine chloride. HiCh can be estimated quantitatively using the hydroxylamine-ferric chloride test [3], and isolated as reineckate or chloroaurate.

Paper chromatography; comparison with the "F component" of oxspleen. HiCh was chromatographed on paper (Fig. 1, A) using the technique recently described [4]. The R_F value was 0.50 compared with acetylcholine (ACh) 0.37, pro-

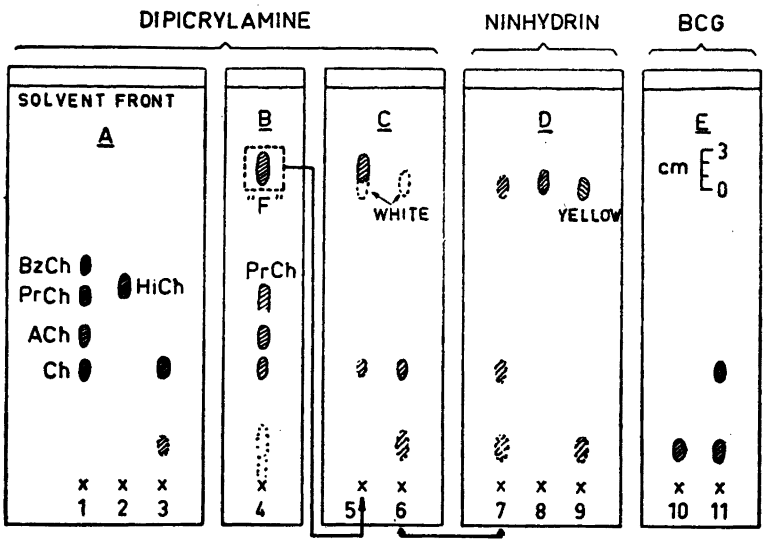

Fig. 1. Ascending chromatograms of hippurylcholine (HiCh) and other choline esters (ACh, acetyl-; PrCh, propionyl-; BzCh, benzoyl-choline), compared with the chromatographic results obtained with the "F component" isolated from ox spleen. Solvent: n-butanol-ethanol-acetic acid-water (8:2:1:3). Development of chromatograms as indicated; BCG, bromocresol green. 1. Choline (Ch) and esters. 2. HiCh. 3. HiCh after alkaline hydrolysis. 4. Isolated bases from spleen. 5. Rechromatogram of "F component". 6 and 7. "F" after alkaline hydrolysis. 8. Hippuric acid. 9. p-aminobenzoic acid. 10. Glycine. 11. Hippuric acid after hydrolysis + choline + glycine.

pionylcholine (PrCh) 0.48, and benzoylcholine (BzCh) 0.55. After alkaline hydrolysis of HiCh, choline (R_F 0.30) was traced on the paper developed with dipicrylamine together with a faintly coloured area (R_F 0.10) which was identified as glycine.

As was mentioned in the introductory section of the present paper, HiCh was assumed to be identical with the "F component" present in ox spleen. The basis for this hypothesis was the following. On the chromatogram of the isolated bases of ox spleen (Fig. 1, B) the "F component" was identified as a yellow spot (dipicrylamine) with the approximative R_F 0.80. The biological activity of this area was verified by testing on the isolated frog rectus abdominis muscle. The "F" area was extracted and rechromatographed (Fig. 1, C). In addition to the "F component" the new chromatogram showed after development with dipicrylamine a white area (R_F 0.77) close to the "F" area and a yellow spot (R_F 0.30) which was identified as choline. After alkaline hydrolysis of the "F component" the original area disappeared and the new areas (R_F 0.77 and 0.30) were more clearly visible; in addition, a third faintly coloured spot (R_F 0.10) appeared. The white area (R_F 0.77) was probably due to an acid compound. By comparing the R_F value of this compound with a great many acid compounds we found *p*-aminobenzoic acid (0.76), *o*-aminobenzoic acid (0.80), *p*-hydroxy benzoic acid (0.84), and hippuric acid (0.77) to come close

Table 1. Effect of hippurylcholine chloride (HiCh) on cholinesterase activity. Corrections made for non-enzymatic hydrolysis.

HiCh *M*	Electric tissue acetylcholinesterase		Human serum cholinesterase	
	b_{30}	% inhibition	b_{30}	% inhibition
Control	68	—	118.5	—
10^{-3}	67	0	13	90
10^{-4}	69	0	57	52
10^{-5}	67.5	0	109.5	8

to the acid compound obtained from the "F component" (Fig. 1, D and E). The slow running compound (R_F 0.10) came close to glycine. The "F component" was therefore assumed to be composed of choline, glycine, and an aromatic acid, presumably an aminobenzoic acid. The working hypothesis was put forward that the new component is a choline ester of a hippuric acid derivative (*e. g.*, *p*-aminohippurylcholine). Choline esters of hippuric acid or its derivatives have not hitherto been described and experiments were therefore performed to synthesize such compounds. Hippurylcholine chloride (HiCh) is the only ester so far synthesized; *p*-aminohippurylcholine has not been obtained in a pure form.

The results obtained by paper chromatography with the "F component" (Fig. 1) demonstrate that hippurylcholine is not identical with this component. The problem to be investigated is whether some derivative of hippuric acid is the acid moiety of the "F component" in ox spleen.

Biochemistry. HiCh is not split enzymatically by the acetylcholinesterase of electric tissue or by a purified preparation of human serum cholinesterase. It has no inhibiting effect in a 10^{-3} *M* solution on the acetylcholinesterase, but inhibits in this concentration the human serum cholinesterase to 90 %. HiCh is regarded as a useful selective inhibitor of the latter enzyme (Table 1).

Pharmacology. HiCh has practically no pharmacological effects when tested on general test objects. No effects were obtained with 1 mg HiCh on isolated guinea-pig ileum, sensitive to 0.1 μg acetylcholine, and on frog rectus abdominis muscle. The compound has no anti-acetylcholine effect.

The blood pressure of the cat is not influenced by 1, 10 and 100 μg HiCh; after 1 mg intravenously a small decrease of the blood pressure was observed, but this effect was probably not specific.

There was no effect on the nictitating membrane, neither stimulating nor inhibiting. The transmission in the superior cervical ganglion of the cat was not influenced after 1 mg HiCh.

HiCh has no antihistaminic effect.

EXPERIMENTAL

β-Chloroethyl hippurate. Hippuric acid (5 g) was dissolved in 2-chloroethanol (10 ml), and hydrochloric acid gas bubbled through the solution for 3 hours. Water was added to the product and when the separated oily phase was mixed with more water a white precipitate was formed. The precipitate was filtered off, washed with water, and dried in a

desiccator. The product was dissolved in hot benzene and precipitated with petrol ether. The pure compound, recrystallized from benzene and dried over P_2O_5, had m. p. 59° C (uncorr.). Yield 74 %. (Found: C 54.88; H 4.97; N 5.78. Calc. for $C_{11}H_{12}O_3NCl$ (241.68): C 54.54; H 5.00; N 5.79.)

Hippurylcholine chloride (HiCh). *a*-Chloroethyl hippurate (1 g) was mixed with a solution (5 ml) containing anhydrous trimethylamine (1 ml) and benzene (4 ml). The mixture was heated in a sealed tube for about 20 hours at 60° C. The oily residue was removed and washed with benzene. An excess of acetone was added to the product which dissolved on heating and crystallized in prisms on cooling. Recrystallization from ethanol (by adding ethyl acetate to the solution) gave white prisms, m. p. 91° C (uncorr.). Yield 25 %. (Found: C 52.94; H 7.27; N 8.77; Cl 10.60. Calc. for $C_{14}H_{21}O_3N_2Cl.H_2O$ (318.80): C 52.75; H 7.27; N 8.79; Cl 11.12.)

Hippurylcholine reineckate, $C_{14}H_{21}O_3N.Cr(NH_3)_2(SCN)_4$. An aqueous solution of HiCh was precipitated with Reinecke salt (aqueous solution). After two hours in the refrigerator the precipitate was filtered off, washed and recrystallized from acetone. The red crystalls had m. p. 135° C (uncorr.).

Hippurylcholine chloroaurate, $C_{14}H_{21}O_3N_2Cl.AuCl_4$, was prepared by a method similar to that used for the reineckate. Recrystallization of the water-insoluble product from acetone gave yellow neddles, m. p. 173° C (uncorr.).

Paper chromatography was carried out according to the method described recently [4,5]. The solvent used was a *n*-butanol-ethanol-acetic acid-water mixture (8:2:1:3). The ascending technique was employed in most cases.

Enzyme and pharmacological studies. Cholinesterase activity was determined by the technique developed in this laboratory [6]. The pharmacological experiments were carried out with test objects in general use.

Isolation of tissue bases was carried out according to the method described recently for the central nervous system of the honey bee [5].

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Studies on Pyrazolones

VIII. 1,1′-Diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione and its Conversion to 1-Phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl)-5-pyrazolone

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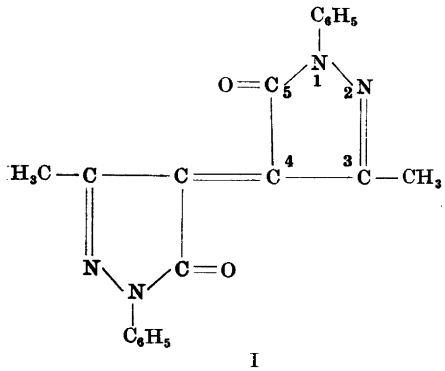
Addition of hydrocyanic acid to pyrazole blue (I) gives 1,1′-diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione (II), which on acid hydrolysis furnishes 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione (III). With alkali, however, 1-phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl)-5-pyrazolone (V) is formed. The mechanism of this rather unexpected reaction is discussed.

Sodium cyanide easily reacts with pyrazole blue (I) at room temperature with the formation of the sodium salt of 1,1′-diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione (II). When the cyano compound is treated with concentrated sulphuric acid, carbon dioxide is evolved and 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione (III) is formed. These reactions can be interpreted in the following way:

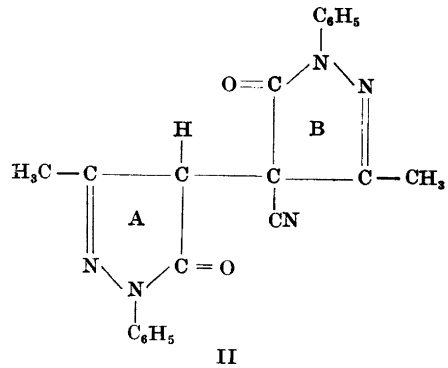
Py = Py → PyHPyCN → PyHPyCOOH → PyHPyH(Py = IV or tautomeric forms).

Treatment of II with alkali and subsequent acidification does not, however, give the expected dibasic acid, III. The product formed is a monobasic acid which similar to III is stable towards boiling, dilute alkali and acids. It has the composition $C_{20}H_{19}N_5O$ and like III forms a salt with two moles of hydrogen chloride. The free compound is obviously 1-phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl)-5-pyrazolone (V). This structure is supported by the fact that in neutral and acid solutions the differences between the light absorption curves of 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione and V are similar to the differences between the curves of 1-phenyl-3-methyl-5-pyrazolone and 1-phenyl-3-methyl-5-aminopyrazole (Figs. 1 and 2).

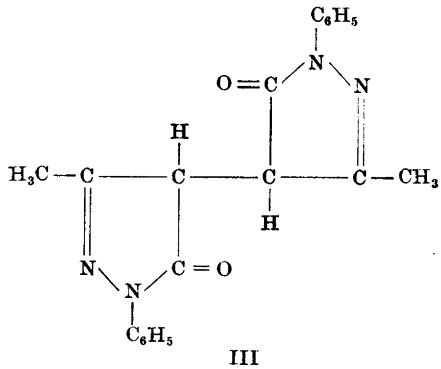
The reactions involved in the transformation of II into V are not immediately obvious. However, the electron attracting C≡N group in the four position of product II is likely to weaken the C_5—N bond of ring B of II, and hence



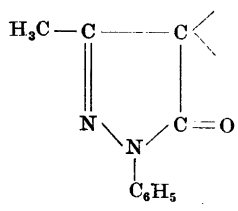
I



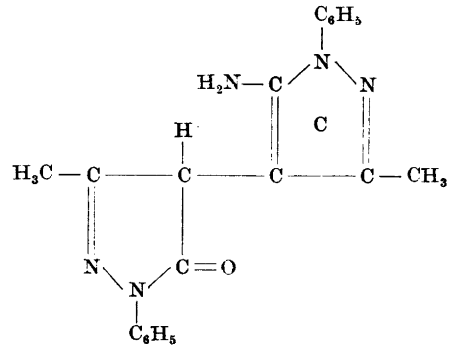
II



III



IV



V

an attractive mechanism is hydrolytic fission, followed by decarboxylation and the formation of a new fivemembered ring (C of V) containing the carbon atom of the C≡N group.

In order to test this hypothesis, 1,1′-diphenyl-3,3′-dimethyl-4-[^{14}C]cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione (II) was prepared by addition of ^{14}C-labelled sodium cyanide to pyrazole blue. When the labelled cyano compound was treated with dilute, carbonate-free sodium hydroxide solution, unlabelled

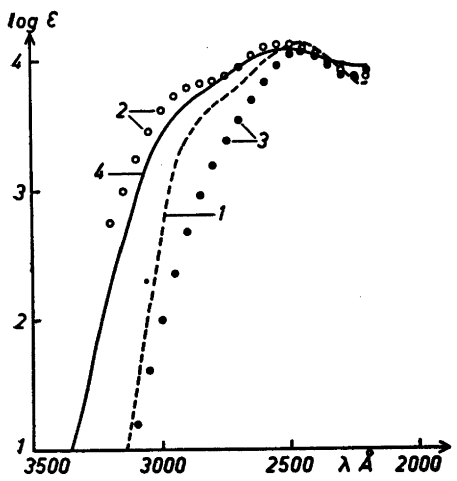


Fig. 1. Light absorption curves of 1-phenyl-3-methyl-5-pyrazolone (1), 1,1'-diphenyl-3,3'-dimethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione (2), 1-phenyl-3-methyl-5-aminopyrazole (3) and 1-phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl)-5-pyrazolone (4) in ethanol.

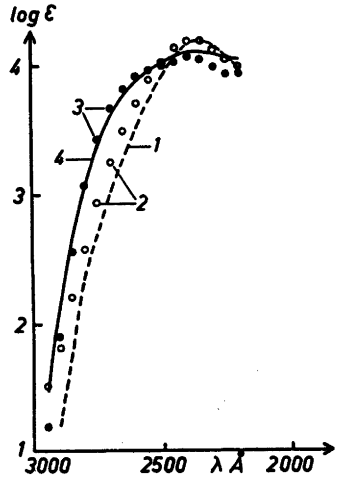


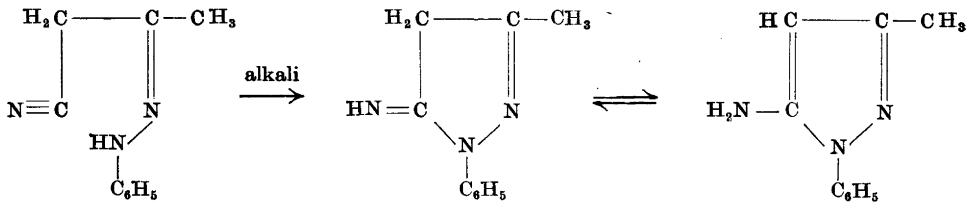
Fig. 2. Light absorption curves of 1-phenyl-3-methyl-5-pyrazolone (1), 1,1'-diphenyl-3,3'-dimethyl-[4,4'-bi-2-pyrazoline]-5,5'-dione (2), 1-phenyl-3-methyl-5-aminopyrazole (3) and 1-phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl)-5-pyrazolone (4) in ethanol with 150 ml of concentrated sulphuric acid per liter.

barium carbonate could be precipitated from the alkaline solution. Precipitation of V with acid yielded a product which contained the same amount of ^{14}C as the cyano compound. Consequently the eliminated carbon atom does not come from the nitrile group, but from a ring. Thus ring B of compound II has been split by alkaline hydrolysis with formation of VIII, and the product VIII has been decarboxylated to form IX in good agreement with the hypothesis.

Cleavage of a pyrazolone ring by alkali was observed by Knorr [1] as early as 1887. He obtained the phenylhydrazone of acetylglyoxylic acid by boiling rubazonic acid with alkali, probably *via* the intermediate 1-phenyl-3-methyl-4,5-pyrazoledione.

The facile decarboxylation in alkaline solution met with above is to be expected, as there are in VIII three multiple linkages in β-γ-position of the carboxyl group (*cf.* the esters of methanetetracarboxylic acid [2] with three double bonds in β-γ-positions and of 1-propene-1,3,3-tricarboxylic acid [3] with two).

Ring-closure of IX to form X probably takes place in the alkaline solution, for after storage overnight a solution of II in *N* methanolic potassium hydroxide has the same UV absorption as a solution of V in the same solvent ($\lambda_{max} = 255$ mμ), whereas neutral solutions of II and V have different absorption curves ($\lambda_{max\ II} = 245$ mμ; $\lambda_{max\ V} = 252$ mμ). Moreover, the phenylhydrazone of cyanoacetone (VI) can be isomerized to 1-phenyl-3-methyl-5-aminopyrazole (VII) by dilute alkali at room temperature.



VI VII

Mohr [4] has shown that the isomerization VI → VII is brought about by warm, dilute acids, and Bell [5] that concentrated hydrochloric acid has the same effect. In the preparation of V only cold, very dilute acid is used for a short time at the final stage, and hence the acid is not likely to cause the ring-closure.

Consequently the formation of 1-phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl)-5-pyrazolone from 1,1′-diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione can be formulated as shown on p. 801.

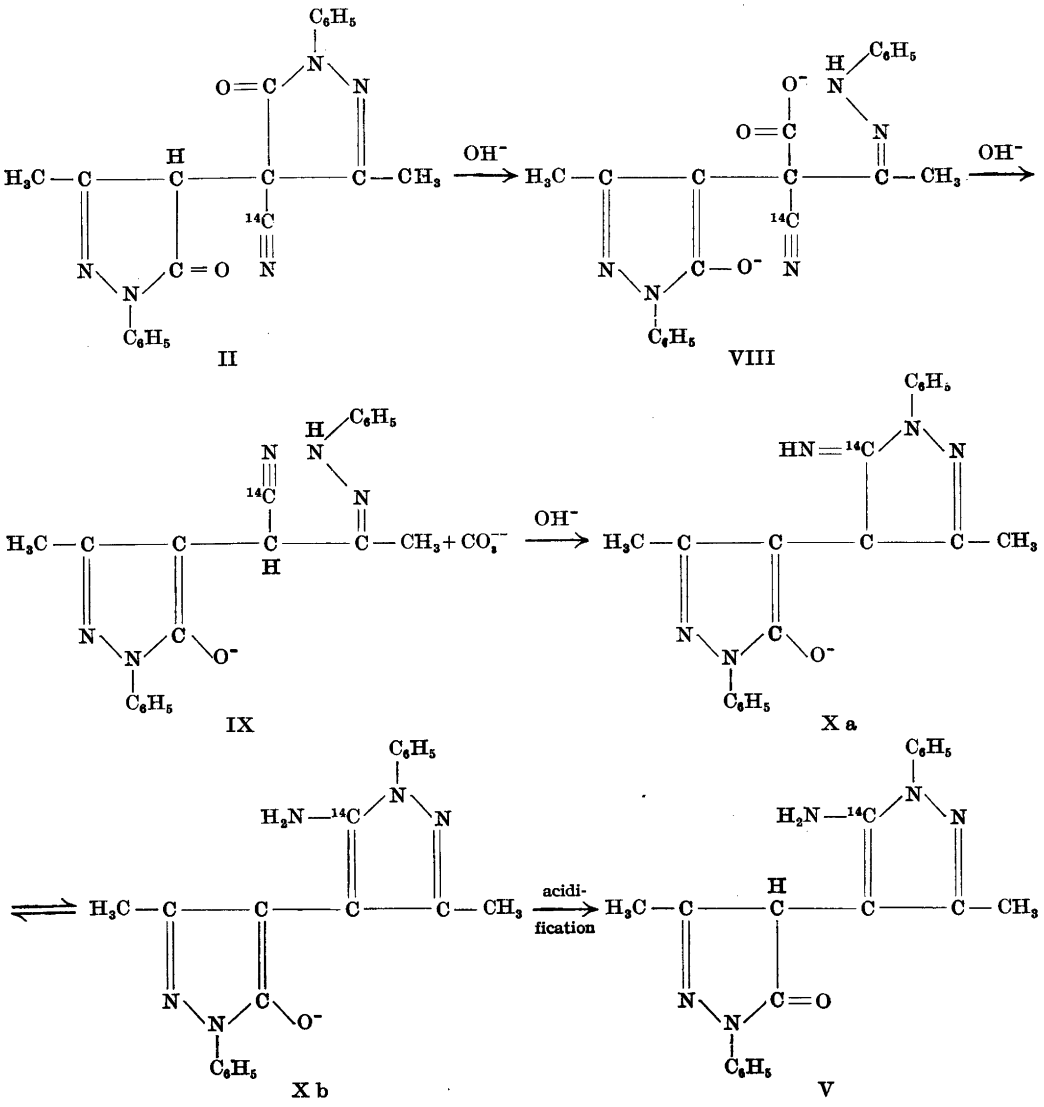
EXPERIMENTAL

1,1′-Diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione (II). Sodium cyanide (1.65 g) in water (5 ml) was added to a suspension of pyrazole blue (7.8 g) in ethanol (10 ml). The mixture was stirred until all the pyrazole blue had reacted (2—3 minutes), when water (250 ml) was added to the red solution. The PyHPyCN formed was precipitated by the addition of acid (10 ml of 4 *N* hydrochloric acid), filtered, washed with water and air-dried.

The product was purified by dissolving in boiling benzene and precipitating with petroleum ether. A small part of the precipitate was transformed into a benzene-insoluble form by heating at 90°—100° C. The rest was dissolved in benzene and seeded with the heated portion. Colourless crystals, soluble in ethanol and acetone, insoluble in benzene, separated. Yield of pure product: 7.5 g (89 %), m. p. 198° (with decomposition). (Found: C 67.8; H 4.8; N 19.0; equiv. wt. 371. Calc. for $C_{21}H_{17}N_5O_2$: C 67.9; H 4.6; N 18.9; equiv. wt. 371.) PyHPy^{14}CN was prepared analogously on a small scale.

Acid hydrolysis of 1,1′-diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione. PyHPyCN (II) (0.79 g) was heated for a few minutes with sulphuric acid monohydrate (5 ml). Carbon dioxide was evolved. The reaction product was precipitated with ice-water, filtered, washed with water and air-dried. The 1,1′-diphenyl-3,3′-dimethyl-[4,4′-bi-2-pyrazoline]-5,5′-dione (0.68 g) formed was purified by extraction of the byproducts with boiling ethanol. It was identified by the equiv. wt., 173, and the formation of pyrazole blue with nitrous acid. PyHPyH was also obtained when the cyano compound was dissolved in concentrated sulphuric acid at room temperature and the solution left overnight.

1-Phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-pyrazolyl) - 5 - pyrazolone (V) by alkaline hydrolysis of 1,1′-diphenyl-3,3′-dimethyl-4-cyano-[4,4′-bi-2-pyrazoline]-5,5′-dione (II). PyHPyCN (2.1 g) was dissolved in 2.5 *N* sodium hydroxide solution (30 ml) and left overnight. Dilute hydrochloric acid was then added. It caused precipitation of the reaction product and impurities. Further addition of acid dissolved the main product but left impurities undissolved. They were removed by filtration, and 0.5 *N* sodium hydroxide was added to the filtrate until precipitation was complete. The precipitate was filtered, washed with water and air-dried. The product was sometimes readily soluble in acetone, sometimes insoluble. In the former case it was purified by solution in acetone, from which crystals soon separated. In the second case purification was simply achieved by extraction with small quantities of acetone. The product can also be purified *via* its hydrochloride. Yield: 1.6 g, m.p. 230—235° with decomposition. (Found: C 69.4; H 5.6;



N 20.1; O 4.70; equiv. wt. 345. $C_{20}H_{19}N_5O$ requires C 69.5; H 5.5; N 20.3; O 4.63; equiv. wt. 345.) 1-Phenyl-3-methyl-4-(1-phenyl-3-methyl-5-amino-4-[5-^{14}C]pyrazolyl)-5-pyrazolone was prepared in the same way.

The hydrochloride was obtained by boiling the substance with concentrated hydrochloric acid. White crystals formed. When heated, hydrogen chloride was liberated, and the substance finally melted at the same temperature as the free aminopyrazolone, about 235°. (Found: C 57.3; H 5.2; N 16.7; equiv. wt. 139.0. $C_{20}H_{19}N_5O$, 2HCl requires C 57.4; H 5.1; N 16.7; equiv. wt. 139.4.)

Measurement of radioactivity. The isotope contents of the ^{14}C-labelled pyrazolones were determined with thin endwindow Geiger-Müller tubes on samples containing less

than 0.1 mg/cm². The samples were prepared by evaporation of ethanol solutions of the products on aluminium planchets. The activity of PyHPy^{14}CN (II) was 7.9×10^9 counts/min./mole, the activity of compound V also 7.9×10^9 counts/min./mole. The ^{14}C-content of barium carbonate, prepared from the carbonate formed during the reaction II → V, was determined on an infinitely thick sample. The activity was negligible.

Isomerisation of cyanoacetone phenylhydrazone with alkali. Cyanoacetone phenylhydrazone was dissolved in ethanol, and 2.5 *N* sodium hydroxide solution was added. The solution was kept overnight, and the product formed was precipitated with water and purified by one crystallization from toluene. It had the same melting point, 115°, and light absorption as an authentic 1-phenyl-3-methyl-5-aminopyrazole. The mixed melting point was unchanged.

Light absorption measurements. In the expression $\log \varepsilon = \log \log \frac{I_0}{I} - \log c \times 1$, used in the diagrams, c is expressed in pyrazole units per liter.

I am indebted to Prof. H. Erdtman and Doc. B. Lindberg for valuable suggestions and to Prof. S. Bergström for the allowance to carry out this investigation at his Institute.

The analyses were carried out by Miss M. Westerdahl, Department of Analytical Chemistry, University of Lund.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 803—806

On Vitamins in Sewage Sludge

III. Vitamin B_{12} Activity of Different Types of Sewage Sludge

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The vitamin B_{12} activity of different types of sewage sludge were studied by means of microbiological estimation and bioautography.

Digested sludge from a plant where the activated sludge process is used was found to have about the same vitamin B_{12} activity per ml (and a considerably higher activity calculated on dry solids basis) but higher percentage of cyanocobalamin than digested sludge from plants where no aeration took place.

Average and limit values are given for the vitamin B_{12} activities of samples of digested sludge collected from a sewage plant during a period of 3 years.

During the bacterial decomposition of sewage sludge, considerable amounts of factors of the vitamin B_{12} group are formed as reported by several authors[1-4]. The values found by different authors for "activated sludge" and for digested sludge vary greatly. There are not only great differences between the values for activated sludge on one hand and digested sludge on the other but also between the values for each kind of sludge. In the present work an attempt was made to investigate whether the type of microbial sludge decomposition has some influence on the amount and kind of vitamin B_{12} activity formed and to follow the vitamin B_{12} activity of digested sludge from a single sewage plant over a longer period of time in order to elucidate whether there exist seasonal variations.

EXPERIMENTAL

Sludge samples from some sewage plants using different methods of sludge treatment were autoclaved at pH 5.5 in the presence of KCN, at 121° C, for 10 min. After centrifugation, a phosphate solution was added to the centrifugate in order to remove calcium ions. The vitamin B_{12}-activity of the centrifugate was then estimated with *Escherichia coli* 113-3 using the cup-plate and tube methods as well as bioautography. Estimations with *Lactobacillus leichmannii* and *Ochromonas malhamensis* have also been made in some cases.

The analyses were made in triplicate and repeated on three or more different days.

RESULTS

Table 1 gives a comparison of the vitamin B_{12} activity of different types of sewage sludge.

I. In the plants Ia, Ib and Ic, digestion of the settled sewage sludge is used but no biological treatment of the effluent. *No aeration* of the sewage takes place at any stage of the treatment. The digestion tanks are provided with stirrers and arrangements for artificial heating (33° C).

II. In plant II the sludge is digested in a large earth basin without stirring or artificial heating. The samples taken at different depths and different

Table 1. Vitamin B_{12}-content of different types of sewage sludge. (For description of the different sewage plants, See text.)

Sewage plant and type of sludge	Number of samples	Average tot. vit. B_{12} activity a)		Average distribution of vit. B_{12}-factors in % of total vit. B_{12}-activity b)			
		mg/l	mg/kg dry solids	Cyano-co-bala-min	Factors A + pseudovit. B_{12} c)	Factor B	Factors C_1 + C_2
Ia							
Fresh sludge	3	0.2	6	40	40	5	20
Digested sludge	27	1.0	14	50	25	5	20
Ib							
Digested sludge	4	0.7	14	50	20	10	20
Ic							
Digested sludge	3	0.9	13	50	20	20	30
II							
Digested sludge	14	(0.9)	(13)	(60)	(30)	(5)	(5)
III							
Digested sludge	2	2.0	25	60	15	5	20
IV							
Fresh sludge	1	0.2	6	50	40	5	5
Digested sludge	3	0.8	25	80	20	—	traces
Activated sludge	3	0.04	11	90	10	—	traces
V							
Digested sludge	1	0.7	11	70	30	—	—
"Biological sludge" (from settling tanks)	1	0.1	3	85	5	5	5
"Scrapes" from biological beds	1	0.3	6	85	5	5	5

a) estimated with *E. coli* 113-3 in the tube-assay

b) after chromatographic separation

c) or possibly also factor III (Bernhauer[4]) which has the same R_F-value as factor A in the chromatographic method applied by us.

distances from the inlet and outlet showed great variations in vitamin B_{12} and dry solids contents. In this case it is not possible to calculate an "average" witamin B_{12} content of the sludge.

III. Plant III receives its sewage from a food factory.

IV. In plant IV the sewage is treated by the activatd sludge process (step aeration).

V. Plant V is equipped with tanks for digestion of sludge and with biological filters for (aerobic) treatment of the effluent from the settling tanks.

DISCUSSION

It can be seen in Table 1 that, *inter alia*, two factors may exert special influence on the vitamin B_{12} activity of decomposed sludge:

1) the nature of the incoming fresh solids.

Thus digested sludge from sewage plant III which receives its sewage from a food factory has a much higher vitamin B_{12} activity (2 mg/l sludge corresponding to 25 mg/kg dry solids) than ordinary municipal digested sludge.

2) the way in which the sewage has been treated.
   viz. a) digestion of settled sludge
   b) digestion of settled sludge in combination with the activated sludge process.

The influence of the first factor has only been investigated in one case. Thus, it is only possible to draw attention to the high vitamin B_{12} activity of food factory sludge — no other comments can as yet be made. The influence of the second factor, however, deserves more attention.

Digested sludge from plants where no part of the sludge has been submitted to aeration before digestion (plants I a, b, c, and II) contains 0.75—1.0 mg/l sludge corresponding to 13—14 mg/kg dry solids of vitamin B_{12} activity. The percentage distribution of the different vitamin B_{12} factors in such a sludge is: cyanocobalamin, about 50 %; factors A + pseudovitamin B_{12}, 15—30 %; factors C_1 + C_2, 20—30 %; factor B, 5—20 %.

Table 2. Vitamin B_{12} activity of digested sludge from sewage plant I a. (Tube assay with E. coli 113-3.)

Total		Average values of 27 samples	Limit values of 27 samples
	mg/l sludge	1.0	0.6 — 1.8
	mg/kg dry solids	14	7 — 30
Cyanocobalamin, % of tot. vit. B_{12} activity		50	15 — 80
Factor A*	—»—	25	10 — 40
Factor B	—»—	5	0 — 30
Factors C_1+C_2	—»—	20	0 — 35

* including pseudovitamin B_{12}.

In the plant V, where the activated sludge process is applied, the sewage is mixed with part of the settled activated sludge and aerated. The excess activated sludge is removed and transferred to digestion tanks *. The digested sludge from this plant has a very high vitamin B_{12} activity when calculated on a dry solids basis: 25 mg/kg solids. Still more surprising is the fact that as much as 80 % of the vitamin B_{12} activity of the sludge is due to cyanocobalamin, factor B being absent and factors C_1 and C_2 occurring only in traces. The high proportion of cyanocobalamin is still more pronounced in the activated sludge where it reaches 90 % of the total vitamin B_{12} activity. In the "biological" sludge and "biological scrapes" from plant V (both derived from aerobic decomposition of organic solids), the corresponding figure is 85 %.

The average values and the variations of the vitamin B_{12} activity of the 27 samples of digested sludge from the sewage plant I a, collected over a period of 3 years, are given in Table 2.

Acknowledgements. The author is greatly indebted to merss. H. Johansson, S. O. Rosenquist, T. Landin, Å. Carlsson, R. Sondén and M. Wennström for kind supply of sludge samples and valuable information on sewage treatment in their respective plants.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 807—809

Low-molecular Carbohydrates in Algae

IX *. Structure of the Glyceric Acid Mannoside from Red Algae

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The structure of the D-glyceric acid α-mannoside occurring in various red algae has been investigated. Methylation, reduction of the methyl ester-methyl ether with lithium aluminium hydride followed by further methylation and hydrolysis yielded a mannose tetramethyl ether and a glycerol dimethyl ether. The two last named substances were shown to be 2,3,4,6-tetra-*O*-methyl-D-mannopyranose and 1,3-di-*O*-methyl-glycerol, and the glycoside is therefore 2-D-glyceric acid α-D-manno-pyranoside.

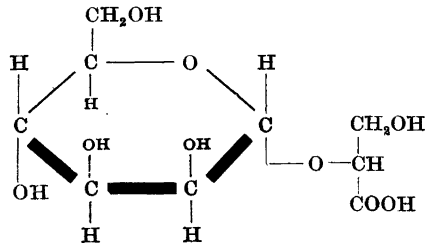
Colin and Augier [1], in 1939, isolated a new glycoside from the red alga *Polysiphonia fastigiata*, which they demonstrated to be an α-mannoside of D-glyceric acid. They assumed the glyceric acid to be linked in 2-position, but gave no experimental evidence for this assumption. Later Augier investigated a large number of red algae and isolated the same glycoside from several of them (Ref.[2] and preceding papers). In the present paper the elucidation of its structure, using the methylation technique, is reported.

The glycoside, isolated as its sodium salt from *Ceramium rubrum*, was methylated with methyl sulphate and sodium hydroxide and the methylated acid esterified with diazomethane. The ester was then reduced with lithium aluminium hydride. A small amount of the reduced product was hydrolysed and investigated by paper chromatography, using as solvent butanol-ethanol-water, 5:1:1, and as reference substances 2,3,4,6-tetra-*O*-methyl-D-mannose, 1-*O*-methyl-glycerol and 2-*O*-methyl-glycerol. Two spots were obtained, identical in R_F-values and colour reactions with the first two reference substances. As expected 2-*O*-methyl-glycerol could not be developed with the reagents used, silver nitrate-sodium ethoxide [3] and lead tetraacetate in benzene [4]. Indications of the presence of 1-*O*-methyl-glycerol in the hydrolysate were thus obtained and this compound should be optically active. In order to obtain inactive

* Part VIII, *Acta Chem. Scand.* **9** (1955) 169.

1,3-di-*O*-methyl-glycerol, which is more easily available as reference substance, the reduced material was further methylated before hydrolysis. The products of hydrolysis were separated by distillation under reduced pressure. The 1,3-di-*O*-methyl-glycerol was characterized as its 3,5-dinitrobenzoate and the 2,3,4,6-tetra-*O*-methyl-D-mannose as its anilide, both being identical with authentic specimens.

According to this study and the previous investigations of Colin and Augier [1], it can be concluded that the structure of the mannoside is 2-D-glyceric acid α-D-mannopyranoside (I).*



I

EXPERIMENTAL

The isolation of the glycoside, as its sodium salt, will be described in a coming publication. Its melting point, 255° (decomp.), and specific rotation, +106° (in water), were in good agreement with the values recorded by Colin and Augier [1].

Methylation of the mannoside. The sodium salt (2.0 g) was methylated with methyl sulphate (11 ml) and sodium hydroxide (9 g) in water (11 ml). The reaction time was 4 hours and the temperature 55–65°. After cooling, the mixture was neutralized with 2 *N* sulphuric acid, diluted with ethanol after slight acidification and the precipitated sodium sulphate was removed by filtration. The solution was neutralized and concentrated and subjected to a second methylation under the same conditions. After neutralization and removal of sodium sulphate as before, the solution was concentrated to a small volume, acidified with sulphuric acid and continuously extracted with chloroform. The chloroform solution was dried over magnesium sulphate and concentrated, yielding a light-coloured syrup (1.87 g).

Esterification and reduction of the methylated product. The methylated product (1.87 g) was dissolved in ether and treated with diazomethane in ether over-night. Excess of diazomethane and ether were evaporated, and the product in dry ether (50 ml) added to a slurry of lithium aluminium hydride (7 g) in dry ether (150 ml). The mixture was vigorously stirred for 2 hours and then water (5 ml) was carefully added. The ether was evaporated, the reaction mixture acidified with 2 *N* hydrochloric acid and extracted with chloroform (3 × 50 ml). The chloroform solution was dried and concentrated, yielding a dark-coloured syrup (1.40 g).

Methylation of the reduced product. The reduced product (1.10 g) was dissolved in dioxan (50 ml), powdered sodium hydroxide (5.5 g) was added and the mixture vigorously stirred for 4 hours at 70–75° C. During the first 45 minutes methyl suphate (6.5 ml) was added in portions. After cooling, the mixture was filtered, the filter cake washed with dioxan and benzene, and the combined filtrates were concentrated and the methylation

* *Added in proof.* Kawaguchi, K., Yamada, S. and Miyama, S. (*Bull. Japan Soc. Sci. Fisheries* **19** (1953) 481. *C. A.* **49** (1955) 4803.) have recently investigated the oxidation of the mannosido-glycerate with lead tetraacetate and arrived at the same conclusion regarding the structure of the substance.

procedure repeated, yielding the crude glycerol mannoside hexamethyl ether as a syrup (1.04 g).

Hydrolysis of the glycerol mannoside hexamethyl ether. The crude hexamethyl ether (1.04 g) was dissolved in 0.5 *N* hydrochloric acid (14 ml), kept at 100° for 22 hours, neutralized by filtering through a column of Amberlite IR 4B, concentrated to a small volume and continuously extracted with chloroform. The chloroform solution was dried over magnesium sulphate and the chloroform evaporated. The product (0.90 g) was fractionated by distillation under reduced pressure (10 mm), yielding 1,3-di-*O*-methyl-glycerol (97 mg, bath temp. 80—100°) and 2,3,4,6-tetra-*O*-methyl-D-mannose (150 mg, bath temp. 125—140°). A considerable residue from the distillation showed that the hydrolysis had not been complete.

Anilide of 2,3,4,6-tetra-O-methyl-D-mannose. (1) A mixture of authentic 2,3,4,6-tetra-*O*-methyl-D-mannose (200 mg), freshly distilled aniline (7 ml) and anhydrous ethanol (7 ml) was refluxed for 8 hours. Ethanol and excess of aniline were evaporated under reduced pressure and the residue crystallized from ethanol to constant m. p., 146—147°. Yield, 40 mg. This procedure is essentially the same as that of Haworth *et al.*[5], but our product has a somewhat higher m. p. (2) The mannose tetramethyl ether from the hydrolysis (40 mg) yielded the same anilide (18 mg), m. p. 146—147°, undepressed on admixture with the authentic material.

3,5-Dinitrobenzoate of 1,3-di-O-methyl-glycerol. (1) A mixture of authentic 1,3-di-*O*-methyl-glycerol [6] (150 mg), 3,5-dinitrobenzoylchloride (310 mg), pyridine (0.2 ml) and Drierite (150 mg) in anhydrous chloroform (2.0 ml) was kept at 50° for 48 hours. The Drierite was filtered off and a small amount of water was slowly added to the mixture followed by ether (10 ml). The solution was extracted successively with water, 1 *N* sulphuric acid, 0.5 *M* sodium hydrogen carbonate and water, and the residue concentrated to dryness. The crude product (204 mg) was crystallized from ethanol to constant m. p., 86—87°. Yield, 60 mg. (Found: N 9.05. Calc. for $C_{12}H_{14}O_8N_2$; N 8.94). (2) The glycerol dimethyl ether from the hydrolysis (97 mg) yielded the same 3,5-dinitrobenzoate (35 mg), m. p. 86—87°, undepressed on admixture with authentic material.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 810—814

Absorption Spectra of Geometrical Isomers of Hexacoordinated Complexes

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The application of the crystal field theory to complexes makes it possible to distinguish between geometrical isomers by a comparison of their absorption spectra. In order to test the theoretical treatment several cobalt(III) complexes of known structure were prepared and their spectra determined. The experimental results were generally found to be in good agreement with the theoretical predictions. Applying this method to *a* and *β*-tris(glycine)cobalt(III), it was possible to show that the *a*-form is the *trans* isomer.

Much of the apparent confusion in the literature with regard to an interpretation of the absorption spectra of *cis* and *trans* complexes of cobalt(III) and chromium(III) appears to have resulted from a failure to recognize the significance of the value of the molar extinction coefficient, ε. It has been shown [1-4] that "the transition group spectra" with an $\varepsilon \lesssim 10^2$ can be explained by applying the crystal field theory to the complex, while the "electron transfer bands" with $\varepsilon \gtrsim 10^3$ can be accounted for by means of Mulliken's LCAO methods [5].

On the basis of crystal field theory nothing can be said about the "third band" of Tsuchida [6] which is reported to occur in all *trans* complexes. The value of the molar extinction coefficient of this "third band" suggests that it is an electron transfer band. It would therefore appear to be accidental if such a band were only to be found in *trans* complexes [7]. On the other hand, all the "low" bands occurring in the visible and near ultraviolet region of the spectra of *cis* and *trans* complexes can be readily treated by means of the crystal field theory.

It is thus somewhat curious that some bands ($\varepsilon \lesssim 10^2$) can be treated as if the complex were "ionic", whereas other bands in the same complex require the assumption that the complex is "covalent" ($\varepsilon \gtrsim 10^3$). It would appear

* On leave of absence from Northwestern University, Evanston, Illinois; John Simon Guggenheim Memorial Foundation Fellow, 1954—1955.

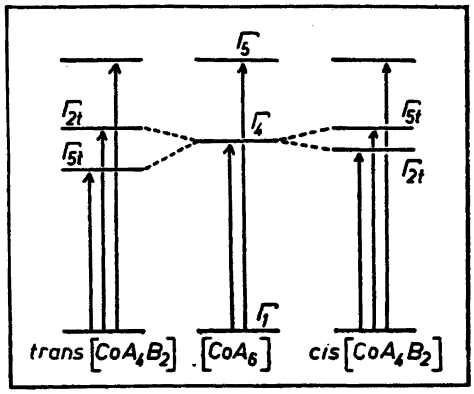

Fig. 1. Transitions responsible for the absorption bands of cobalt(III) complexes with cubic $[CoA_6]$ *and with tetragonal* $[CoA_4B_2]$ *crystal fields.*[3]

that such a difficulty arises from the fact that both points of view are merely approximations. Furthermore, it should be made clear that the considerations of the crystal-field theory do not support the distinction made between "ionic" and "covalent" complexes in terms of their magnetic susceptibilities [1]. The magnetic criterion as a guide to actual bond type in complexes is therefore somewhat limited.

Ballhausen and Klixbüll Jørgensen [3] have treated the *cis* and *trans* isomers of hexacoordinated complexes from the point of view of the crystal field. It was shown that if the absorption bands of a cubic complex $[MA_6]$ were split up by superimposing a crystal field of lower symmetry upon the metal ion, then the splitting of a *trans* complex $[MA_4B_2]$ would be twice the splitting of the same *cis* isomer. That this is approximately what has been observed can be seen from the spectra given by Linhard and Weigel [8] as well as from the curves in this paper.

The complexes investigated and discussed here are cobalt(III) complexes. This choice was made merely because several of these complexes of known structure can easily be prepared and their spectra determined. Orgel [4] has shown that the two absorption bands of luteo cobalt(III) complexes $[CoA_6]$ are due to $\Gamma_1 \rightarrow \Gamma_4$ and $\Gamma_1 \rightarrow \Gamma_5$. Further that to a first approximation whenever the cubic crystal field of the luteo complex is altered to a tetragonal field only the first band is split (Fig. 1). These splittings are determined by the sum of the contributions along each axis. However, the splittings are apparent only when an appreciable difference exists between these contributions.

A qualitative estimate of this difference is furnished by the spectrochemical series originally developed by Fajans [9] and Tsuchida [6],

$$I^- < Br^- < Cl^- \lesssim OH^- < RCOO^- < NO_3^- < F^- \lesssim H_2O \lesssim SCN^- < NH_3 < en$$
$$\lesssim NO_2^- < o\text{-phen} << CN^-.$$

Orgel [4] has shown that if in a complex of the type CoA_4B_2, the substituent B is placed to the left of A in the above series, then for the split band of the *trans*

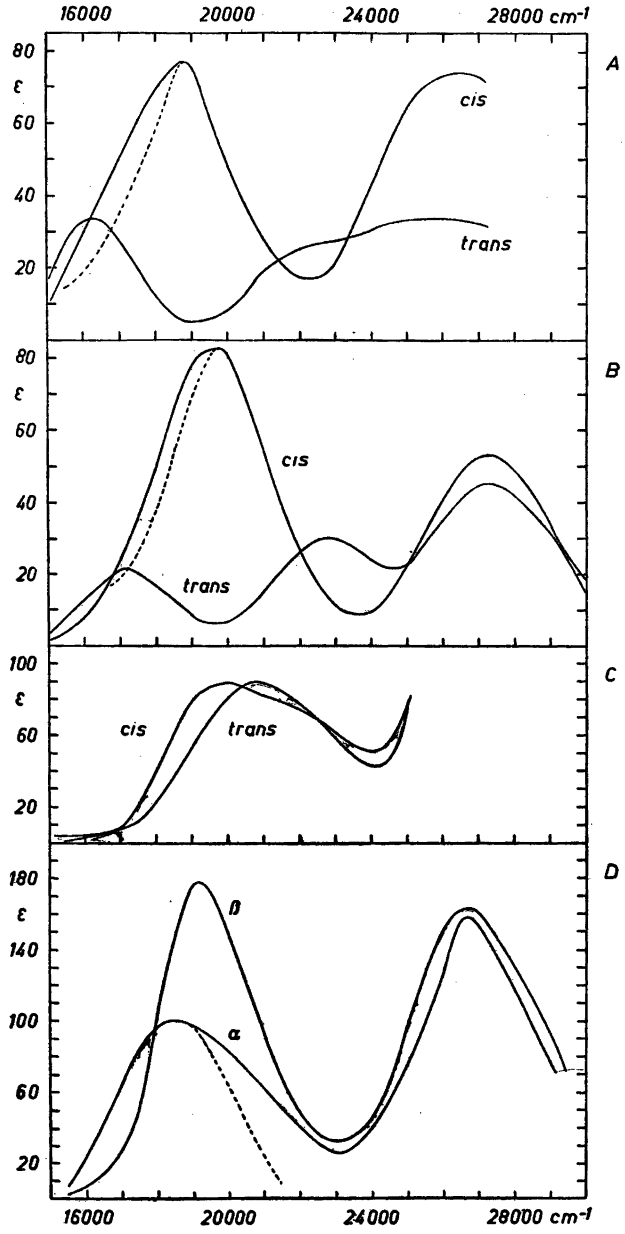

Fig. 2. Molar extinction coefficient ε vs *wavenumber in* cm^{-1}.

A: *cis*-[Co en_2Cl_2]Cl 0.02015 *M*
tr.-[Co en_2Cl_2]Cl 0.01672 *M*
B: *cis*-[Co en_2F_2]NO_3 0.0177 *M*
tr.-[Co en_2F_2]NO_3 0.0184 *M*
C: *cis*-[Co $en_2(NO_2)Cl$]Cl 0.01617 *M*
tr.-[Co $en_2(NO_2)Cl$]NO_3 0.00771 *M*
D: α-[$Co(NH_2CH_2COO)_3$], $2H_2O$ 0.00188 *M*
β-[$Co(NH_2CH_2COO)_3$], H_2O 0.000511 *M*

complex the long wavelength component will be twice as intense as the short wavelength component. If B is placed to the right of A then it is the short wavelength component of the band which is going to be twice the long wavelength component. For a *cis* complex of this type, the reverse of the above arguments applies.

Further, it can be shown that when the complex under discussion has a center of symmetry as $[MA_6]$ and *trans*-$[MA_4B_2]$, the total area under the bands, which gives a measure of the intensity of the absorption, is smaller than if there is no center of symmetry (*cis*-$[MA_4B_2]$). It follows that the intensity of a *cis*-compound MA_4B_2 should be greater than that of the *trans* compound. For complexes in which neither geometrical isomer possesses a center of symmetry ($[MA_4BC]$ and $[MA_3B_3]$), both *cis* and *trans* compounds will have approximately the same area under the absorption curves. That this is the case can be seen in the plots shown in this paper.

EXPERIMENTAL

Preparation of compounds. The compounds investigated were prepared by methods described in the literature as previously reported [7].

The tris(glycine)cobalt(III) was prepared in two forms by Ley and Winkler [10]; a violet soluble form (α) with two molecules of water, and a red nearly insoluble form (β) with one water of crystallization. They are supposed to be *cis* and *trans* isomers of the type $[MA_3B_3]$, but it has not been definitely established as to which has the *cis* and which the *trans* structure.

The *cis* and *trans* isomers of $[Co\ en_2F_2]NO_3$ were kindly supplied by W. R. Matoush, who will soon publish the method of synthesis and properties of these complexes in the Journal of the American Chemical Society.

Measurements. All measurements were made with a Cary spectrophotometer using 1 cm corex cells, and 10 cm cells for the most dilute solutions. The solvent was water and dilute nitric acid solution in case of aquo complexes. The cobalt concentrations of the solutions were between $\sim 10^{-2}$ and $\sim 10^{-3}$ *M*. Measurements were made at room temperature and a total operation time of less than ten minutes was required, starting from the time the water was added to the solid salt. Only in the case of *trans*-$[Co\ en_2(NO_2)Cl]^+$, which aquates fairly rapidly [11], was it necessary to determine the spectrum at three different times and extrapolate back to zero time in order to get the spectrum of the chloro complex.

DISCUSSION OF RESULTS

Some examples which show that the absorption spectra found are in good agreement with the predictions, are given in Fig. 2. Plots A and B ($[Co\ en_2Cl_2]^+$ and $[Co\ en_2F_2]^+$, respectively) demonstrate (1) that the displacements of the bands follow the spectrochemical series, and (2) that the splitting of *trans* isomers of this type is approximately twice that for the same *cis* isomer. It is further observed (3) that the areas under the curves are less for the *trans* isomers, which have a center of symmetry, than for the same *cis* isomers which do not have this property.

Considering that F^- and H_2O are very close together in the spectrochemical series, it was expected that the spectra of the diaquo complexes should resemble the spectra of the difluoro complexes. That this is the case has been demonstrated by Bjerrum and Rasmussen [12]. On the other hand we cannot understand why the first band of the hydroxo complexes [12] does not show any splittings.

Plot C gives the absorption spectra for isomers of the type $[MA_4BC]$. The available data show that too much emphasis must not be placed on the "absolute" positions of the ligands in the spectrochemical series in complexes with increasing number of different ligands. For the complex considered $[Co\ en_2(NO_2)Cl]^+$, a splitting of the band is observed with the *cis*, but not with the *trans* isomer. According to the theory the splitting is determined by differences in the sum of the crystal field contributions along each axis [3].

Because of the fact that the contribution of the chloride ion is much less than that of $\frac{1}{2}$en and NO_2^- the configuration of the *cis* isomer is approximately tetragonal. Since in the *trans* isomer the tetragonal axis is more similar to the other axis than in the *cis* isomer, splitting should be expected to occur in the *cis* rather than in the *trans* complex.

Since neither of these isomers has a center of symmetry, the areas under their curves are approximately equal.

Geometrical isomers of $[Co\ en_2(NO_2)_2]^+$, $[Co\ en_2(NCS)_2]^+$, $[Co\ en_2NO_2NCS]^+$ and $[Co\ en_2(H_2O)NO_2]^{++}$ were also investigated. Neither the *cis* nor the *trans* isomers of the dinitro, dithiocyanato or the nitrothiocyanato complexes showed any splitting. This is attributed to the fact that these ligands are not too "different", therefore the sum of the contributions along each axis in the complexes are approximately the same. However, the absorption spectrum of *cis*-$[Co\ en_2(H_2O)NO_2]^{++}$ shows a slight splitting contrary to that of the *trans* isomer; these results are similar to the chloronitro complex.

Finally on the basis of the curves in Fig. 2 D, it is believed that the *trans* configuration can be assigned to the α-tris(glycine)cobalt(III) complex. The absorption band of this isomer is clearly split in accord with a rhombic crystal field, whereas that for the β-form is not split as expected for a cubic crystal field.

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The Use of the Electron Diffraction Sector Method for Location of Hydrogen in Gas Molecules

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Molecular structure determination of gaseous ammonia and ethane has been carried out with the aid of the electron diffraction sector method. The structure parameters found are the following: Ammonia, N—H = 1.015 Å and ∠H—N—H = 106.6° ± 4.0°. Ethane, C—H = 1.107 Å, C—C = 1.536 Å and ∠C—C—H = 109.54°. The ethane molecule shows the *trans* form.

Structural chemists have tried in recent years to improve the various methods for determination of the position of hydrogen atoms in crystal lattices and in free gas molecules [1]. To investigate the possibilities of the electron diffraction sector method for this kind of study we have chosen two rather simple and structurally well-known molecules, namely ammonia and ethane.

The electron diffraction apparatus used has been described earlier [2].

Ammonia was obtained from ammonium chloride and dry slaked lime. The pictures were taken using an ordinary liquid-air trap. Better results might have been obtained by the procedure described below for ethane. Intensity data for large s values were not obtained, the data extend only to about $s = 30$ Å^{-1}. The following general procedure is applied to obtain the best intensity and radial-distribution curves: The microphotometer curves are read off in a logarithmic scale, correction for the deviation from linearity of the density values is carried through, and the resulting curve is multiplied by the factor

$$c\,\frac{s^4}{\alpha\cos^3 2\Theta}$$

where α is the sector-screening factor and $\cos^3 2\Theta$ takes care of the correction necessary because of the use of plane photographic plates. The constant c is chosen to make the resulting curve (I_E) fit the scale of the background

$$B_T = \sum_i [(Z_i - F_i)^2 + S_i]$$

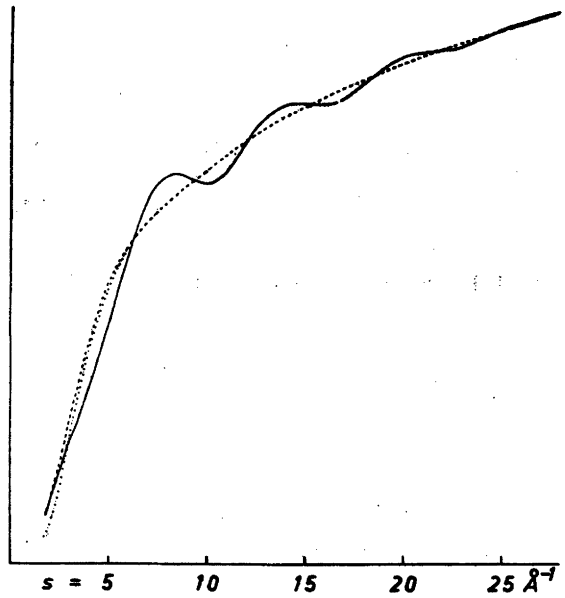

Fig. 1. The I_E *curve of ammonia with background drawn in.*

Though this procedure in most cases gives a fairly good fit of the intensity curve to the background, it is usually necessary to draw an empirical background for at least some parts of the s range. In Fig. 1 the fully-drawn curve is the inner part of the I_E curve. The dashed curve is the background as drawn in. The background is subtracted from the I_E curve and the difference is multiplied by the factor

$$\frac{s}{\left(1-\frac{F'}{Z}\right)^2} e^{-ks^2}$$

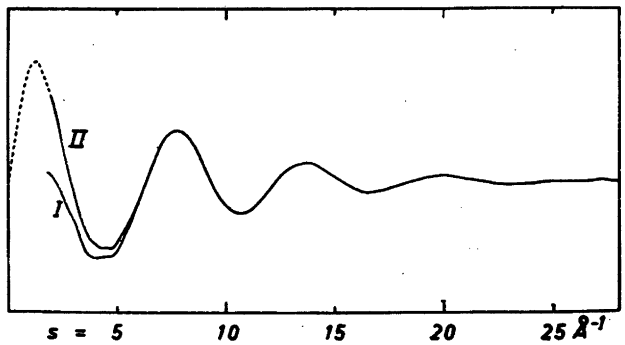

Fig. 2. Intensity curve of ammonia.

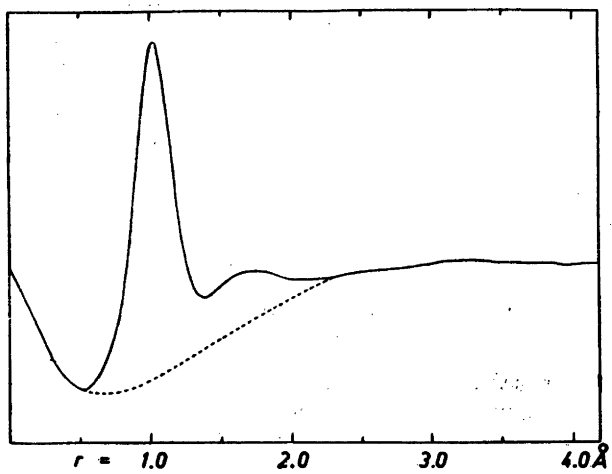

Fig. 3. Radial distribution curve of ammonia with "envelope".

k being in this case 0.0036, and $\left(1-\frac{\bar{F}}{\bar{Z}}\right)^2=\left(1-\frac{F_N}{Z_N}\right)\left(1-\frac{F_H}{Z_H}\right)$. The curve thus obtained is given as curve I in Fig. 2. The Fourier transform of this curve is shown in Fig. 3.

Because of experimental limitations we cannot obtain the intensity values of the first ring from $s = 0$ to about $s = 1.5$ Å^{-1}. Further, the empirical background introduces uncertainties in the subtracted intensity curve, particularly in the inner part of the curve. To allow for these defects we draw in an "envelope" in the radial distribution curve; dashed curve in Fig. 3. This envelope coincides with the radial distribution curve in the areas $0 < r < 0.6$ and $2.2 < r$. The difference between the original radial distribution curve and the envelope is shown in Fig. 4 (fully-drawn curve). The Fourier transform of the

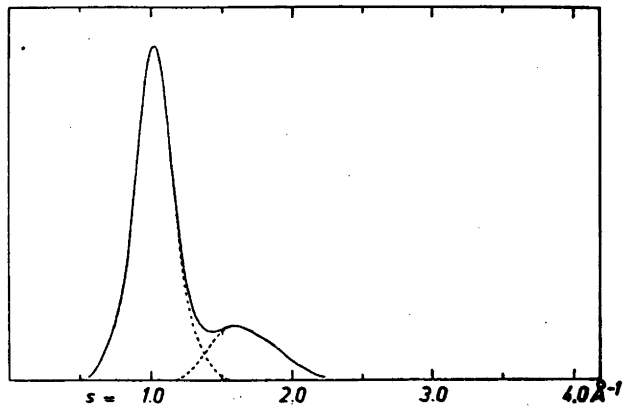

Fig. 4. Radial distribution curve of ammonia. Envelope subtracted.

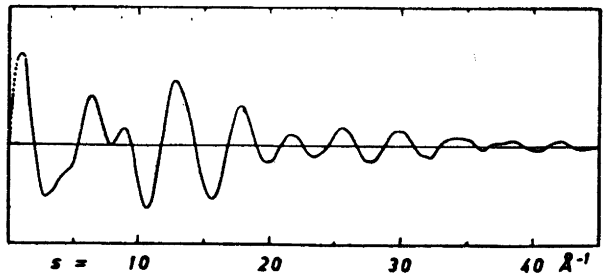

Fig. 5. Intensity curve of ethane.

envelope has also been calculated and added to the intensity curve I of Fig. 2, giving curve II of the same figure. This curve now, of course, shows the inner peak which could not be determined directly from the experiments. It has also been corrected somewhat in the area from $s = 1.5$ to $s = 6$ Å^{-1}. This correction corresponds to only a slight change in the background. The new background is shown as the dotted curve in Fig. 1.

Two more radial distribution curves have been calculated using different k values in the exponential factor e^{-ks^2} ($k = 0$ and $k = 0.0015$). The maximum of the main peak corresponding to the N—H distance ($k = 0$) occurs at 1.015 Å, in excellent agreement with the spectroscopically obtained value[3] of 1.014 Å.

The H—H distance shows up as a well-resolved peak both in Fig. 3 and in Fig. 4. In Fig. 4 the contribution of the N—H peak is subtracted to give the shape of the H—H peak. This does not have the usual symmetric form of radial distribution peaks. This might be due either to experimental imperfections or to the conversion mechanism of the ammonia molecule. The maximum of the H—H peak occurs at $r = 1.584$ Å, corresponding to an H—N—H angle of 102.6°. The average r value found from the "center of gravity" of the peak is 1.668 Å, corresponding to an angle of 110.5°. It is a reasonable assumption to believe that the equilibrium distance is somewhere between these values. Our determination should, accordingly, lead to an angle of 106.6° ± 4°, which is in good agreement with the spectroscopically obtained value of 106.8° [3].

Ethane was prepared by electrolysis of sodium acetate dissolved in glacial acetic acid. The cold trap of the electron diffraction apparatus was cooled by liquid air evaporated under reduced pressure. Under these circumstances, we obtained a temperature on the liquid-air trap of approximately —210°, at which temperature the vapour pressure of ethane was low enough not to destroy the vacuum in the electron diffraction apparatus.

Fig. 5 is the final undamped intensity curve corrected in the same way as described for ammonia. Since the outer part of the intensity curve contributes very little to the non-bonded distances of special interest in this work, no particular effort was done to obtain intensity data for the very large s values. On the other hand, our diagrams which extend to $s = 45$ Å^{-1} do not give any indication of diffraction rings much beyond this point. Four radial distribution curves were calculated. One of them was based upon an undamped intensity curve, the other ones were based upon intensity curves multiplied by e^{-ks^2},

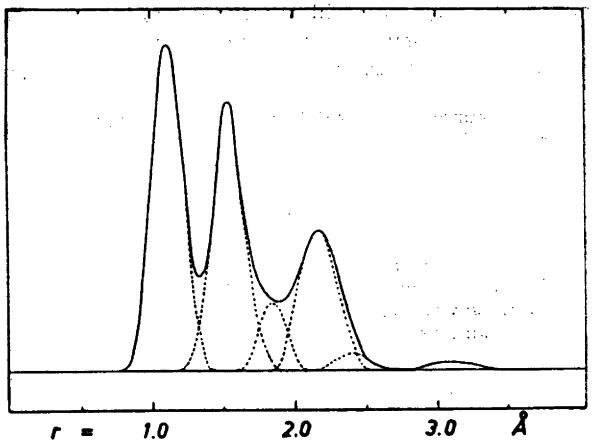

Fig. 6. Radial distribution curve of ethane.

k ranging from 0.0015 to 0.003. (For Fig. 6, $k = 0.003$). The three predominant peaks of the radial distribution curves correspond to the C—H bond, the C—C bond, and the long C···H distance. The best values obtained for these three distances are 1.107, 1.536, and 2.173 Å, respectively. This leads to ∠ C—C—H = 109.54° and ∠ H—C—H = 109.40°. These values are in fair agreement with the values obtained from spectroscopic measurements by Hansen and Dennison [4] who find C—H = 1.102 Å, C—C = 1.543 Å, and ∠ C—C—H = 109.62°.

If we assume the *trans* form for the ethane molecule, we find that three different H—H distances should exist. One of them, the *trans* distance, is easily determined as the corresponding peak occurs at an r value well beyond the region of the peaks of the three predominant distances. The two other H—H distances, however, must be determined indirectly. This is done by assuming the peaks from each distance to be symmetrical. In this way the right side of the first peak is easily constructed and subtracted from the radial distribution curve. Then the left side of the second peak is constructed. By continuing this procedure the two remaining H—H peaks are found. The first H—H peak, corresponding to the H···H distance through one angle, can be determined quite accurately. The correct shape of the second H···H peak corresponding to the *gauche* distance, however, is more difficult to find in this simple manner, because the various necessary corrections introduce uncertainties. A closer analysis of these corrections, however, seem to establish beyond doubt the existence of the *gauche* H—H contribution. In the final determination of the *gauche* peak, the C···H peak has been given the proper weight before subtraction, *i. e.* the area of the C···H peak is made equal to that of the first C—H peak multiplied by 1.107/2.173.

The three H—H distances thus obtained are the following: the H—H distance through one angle 1.83 Å, the *gauche* distance 2.44 Å, and the *trans* distance 3.09 Å. The corresponding distances calculated using the parameters

given above are 1.81, 2.50, and 3.09, respectively. Thus, even if the *gauche* distance is not determined very accurately we may consider the present studies as a direct proof for a predominating *trans* form in the gaseous ethane molecule.

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On some Intensity Problems in the Absorption Bands of Complex Ions

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A qualitative estimate of the intensities in the absorption bands of complex ions may be obtained by a consideration of the symmetry properties of the complex. Special attention is directed (1) towards the vibrations of the ligands and (2) towards the selection rules for electric dipole transitions. These points of view are applied to the bands of some simple complexes of the first transition group.

A theory for the intensities of the absorption spectra of complex ions has been given by Van Vleck [1] and by Broer, Gorter and Hoogschagen [2]. It is the intention here to study the application of the theory set forth by these authors to the absorption bands of some simple complexes of the first transition group.

Assuming [3] that the absorption bands may be described as Gaussian curves, the probability of absorption P can be found as

$$P = 4.60 \cdot 10^{-9} \cdot \varepsilon_0 \cdot \nu_{1/2} \tag{1}$$

ε_0 being the molar extinction coefficient of the maximum and $\nu_{1/2}$ being the halfwidth of the band measured in cm^{-1}.

The quantum mechanical expression for P is [1,2]

$$P = \frac{8\pi^2 mc}{3h} \nu \, M_{I,II}$$

$$M_{I,II} = \underset{I}{av} \sum_{II} \left| \int \psi_I \vec{r} \, \psi_{II}^* \, d^3r \right|^2 \tag{2}$$

ν is the position of the maxima of the absorption bands in wavenumbers and $M_{I,II}$ is the average over initial states I, and sum over the final states, II, of the squares of the matrix elements of the electric dipole vector $\vec{r}$. m and c are the mass of the electron and the speed of light, and h is Planck's constant.

It has been shown [4-6] that the absorption bands of complexes of the first transition group are due to transitions between the various levels in which the undisturbed terms of the metal ion split up under the influence of the ligand field. The resulting levels may be classified according to their Γ number [7]. In a cubic complex (point group O) these numbers, which specify the symmetry properties of the level, go from Γ_1 to Γ_5. If a complex of the first transition group has a center of symmetry all the lowest levels are "even" under reflections in space. It follows then from Laportes rule that $M_{I,II}$ is equal to zero. In a cubic complex MA_6 the dipole absorption is thus completely forbidden. However, as pointed out by Van Vleck [1], dissymmetry caused by vibrations of the ligands demolishes the existence of such a center of symmetry. In a nonsymmetric complex the "even" wavefunctions coming from the d^n configurations can intermix with the "odd" wavefunctions of the $d^{n-1}p$ terms. This means that the perturbed wavefunctions are neither "even" nor "odd" and Laportes rule cannot be applied. That the existence of a symmetry center really is of significance for the intensities of the bands has been demonstrated experimentally by Basolo, Ballhausen and Bjerrum [8].

This is, however, only one side of the question. For pure symmetry reasons dipole transitions are only allowed between the following levels:

$$\Gamma_1 \rightarrow \Gamma_4;\ \Gamma_2 \rightarrow \Gamma_5;\ \Gamma_3 \rightarrow \Gamma_4 \text{ and } \Gamma_5;\ \Gamma_4 \rightarrow \Gamma_1, \Gamma_3, \Gamma_4 \text{ and } \Gamma_5;\ \Gamma_5 \rightarrow \Gamma_2, \Gamma_3, \Gamma_4 \text{ and } \Gamma_5.$$

These selection rules become more and more important the stronger the field. This is due to the general trend that the stronger the field, the better the quantum number Γ_n. These last selection rules are therefore rather important for Cr(III) and Co(III) complexes, but are of less importance for Ti(III), V(III), Co(II) and Ni(II) complexes. It must further be emphasized that the above discussion applies only to complexes with cubic symmetry.

Van Vleck was mainly interested in the intensity problems for the absorption bands of the rare earths. However, the results of Van Vleck [1] may also be used in this connection. The ligands are then supposed to be placed in a potential hole of the shape $V = \frac{\beta}{r^6} - \frac{Z\mu}{r^2}$, where β is a constant, μ the value of the electric dipole moment of the ligand, Z is the charge of the metal ion and r the distance. The intensity due to vibrations of the ligands is then [1]:

$$P = \nu \left(\frac{\pi_{hol}}{\nu^+}\right)^2 \cdot e \cdot \frac{r_o^2}{\sqrt{\mu Z M}} 10^{-7} \qquad (3)$$

ν^+ is the distance to the nearest "odd" level, π_{hol} is the holoedric crystal field, e the degeneracy of the upper level, M the molecular weight of the ligand and r_o the equilibrium distance of the ligands. It is difficult to estimate a value of π_{hol}. For the purpose here it is set equal to $2(E_1 - E_2)$. r_o is equal to 2 Å and $\sqrt{\mu Z M} \sim 16$, μ being measured in Debye units.

Then:

$$P_\nu = \nu \cdot \left(\frac{E_1 - E_2}{\nu^+}\right)^2 \cdot e \cdot 10^{-7} \qquad (4)$$

Further if there are several bands in a complex, the ratio between the absorption probabilities of different bands may be taken, thereby obtaining a formula which does not contain the more uncertain factors of (3):

$$\frac{P_{\nu n}}{P_{\nu m}} = \frac{\nu_n \cdot e_n}{\nu_m \cdot e_m} \qquad (5)$$

Ti^{+++} $3d^1$. $\nu^+ = 13 \cdot 10^4$ cm^{-1}.
$(E_1—E_2)_{aq} = 20\,000$ cm^{-1}. $\nu_1 = 20\,000$ cm^{-1}.
$P_{\nu 1}$ found[9]: $1.6 \cdot 10^{-4}$ $P_{\nu 1}$ calc.: $1.0 \cdot 10^{-4}$

V^{+++} $3d^2$ $\nu^+ = 15 \cdot 10^4$ cm^{-1}
$(E_1—E_2)_{aq} = 20\,500$ cm^{-1}. $\nu_1 = 17\,200$ cm^{-1} $\nu_2 = 25\,000$ cm^{-1}
$\nu_3 = 37\,000$ cm^{-1} (calc.)

$P_{\nu 1}$ found[10]:	$9.3 \cdot 10^{-5}$	$P_{\nu 1}$ calc.:	$10 \cdot 10^{-5}$
$P_{\nu 2}$ — — —	$18 \cdot 10^{-5}$	$P_{\nu 2}$ — — —	$14 \cdot 10^{-5}$
$P_{\nu 3}$ — — —	?	$P_{\nu 3}$ — — —	$6 \cdot 10^{-5}$

Co^{++} $3d^7$ $\nu^+ = 10 \cdot 10^4$ cm^{-1}
$(E_1—E_2)_{aq} = 9\,000$ cm^{-1}. $\nu_1 = 8\,000$ cm^{-1} $\nu_2 = 16\,000$ cm^{-1}
$\nu_3 = 20\,200$ cm^{-1}

$P_{\nu 1}$ found[11]:	?	$P_{\nu 1}$ calc.:	$2.0 \cdot 10^{-5}$
$P_{\nu 2}$ — — —	$\sim 0.1 \cdot 10^{-5}$	$P_{\nu 2}$ — — —	$1.2 \cdot 10^{-5}$
$P_{\nu 3}$ — — —	$9 \cdot 10^{-5}$	$P_{\nu 3}$ — — —	$5.0 \cdot 10^{-5}$

Ni^{++} $3d^8$ $\nu^+ = 11 \cdot 10^4$ cm^{-1}.
$(E_1—E_2)_{aq} = 7\,600$ cm^{-1}. $\nu_1 = 8\,000$ cm^{-1}. $\nu_2 = 14\,000$ cm^{-1}
$\nu_3 = 25\,300$ cm^{-1}.

$P_{\nu 1}$ found[12]:	$2 \cdot 10^{-5}$	$P_{\nu 1}$ calc.:	$1.2 \cdot 10^{-5}$
$P_{\nu 2}$ — — —	$3.5 \cdot 10^{-5}$	$P_{\nu 2}$ — — —	$2.0 \cdot 10^{-5}$
$P_{\nu 3}$ — — —	$7.2 \cdot 10^{-5}$	$P_{\nu 3}$ — — —	$3.6 \cdot 10^{-5}$

$\frac{P_{\nu 1}}{P_{\nu 2}}$ found: 0.57 calc.: 0.60

$\frac{P_{\nu 2}}{P_{\nu 3}}$ found: 0.49 calc.: 0.55

$(E_1—E_2)_{en} = 10\,500$ cm^{-1} $\nu_1 = 11\,200$ cm^{-1} $\nu_2 = 18\,350$ cm^{-1}
$\nu_3 = 29\,000$ cm^{-1}.

$P_{\nu 1}$ found[12]:	$9.0 \cdot 10^{-5}$	$P_{\nu 1}$ calc.:	$3.0 \cdot 10^{-5}$
$P_{\nu 2}$ — — —	$10.2 \cdot 10^{-5}$	$P_{\nu 2}$ — — —	$5.0 \cdot 10^{-5}$
$P_{\nu 3}$ — — —	$14.2 \cdot 10^{-5}$	$P_{\nu 3}$ — — —	$8.0 \cdot 10^{-5}$

$\frac{P_{\nu 1}}{P_{\nu 2}}$ found: 0.88 calc.: 0.60

$\frac{P_{\nu 2}}{P_{\nu 3}}$ found: 0.70 calc.: 0.62

$\frac{P_{\nu 3(aq)}}{P_{\nu 3(en)}}$ found: 0.50 calc.: 0.45

However, in formula (5) the selection rules of the Γ's are not considered.

Some examples taken from the literature will now be compared with the theory. Due to the points previously discussed only cubic MA_6 complexes which can be treated from the standpoint of the weak crystal field will be considered. This leaves out Cr(III) and Co(III) where the selection rules of the Γ's are important, and Cu(II) complexes which have a distinctly tetragonal structure [4, 13].

The values of ν^+ have been taken from "Atomic Energy Levels" (National Bureau of Standards 467 (1949)).

It is seen, that while the absolute values of P calculated by means of (4) are of the right order of magnitude, the intensity ratios determined by means of (5) are, at least for the Ni(II) complexes, in much better agreement. However, the part played by the selection rules can be clearly seen in Ref.[3], Fig. 5. The second band in the spectrum of $Nien_3^{++}$ due to a $\Gamma_2 \rightarrow \Gamma_4$ transition has a smaller intensity than the first band which is due to a $\Gamma_2 \rightarrow \Gamma_5$ transition. The same explanation may account for the discrepancy found for P_{ν_2} in Co(II) complexes.

P is seen to be proportional to $\varepsilon_0 \cdot \nu_{1/2}$. As pointed out by Bjerrum, Ballhausen and Klixbüll Jørgensen [13], and as shown by Orgel [14] $\nu_{1/2}$ is proportional to the slopes of the energy levels for the transition in question. This means, all other things being equal, that if the differences in the slopes are great and consequently $\nu_{1/2}$ large, then the molar extinction coefficient are likely to be small.

The picture of the complex molecule as a metal ion surrounded by point dipoles is of course very crude, and one must expect that exchange phenomena *etc.* will play an important role for a detailed description. It seems however that the main features of the intensities may be described within the simple picture offered by the crystal field theory.

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The Influence of the Solvent on Reaction Velocity

XI. Alkaline Hydrolysis of Ethyl Bromide in Acetone-Water Mixtures

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In contrast to the uncatalysed hydrolysis of alkyl halides, the reaction between ethyl bromide and hydroxyl ion, $EtBr + OH^- \rightarrow EtOH + Br^-$, in acetone-water is little affected by changes in the proportion of water in the solvent. The specific reaction rate increases slightly with an increasing proportion of acetone up to 18—27 wt.% acetone; there is a marked decrease of the rate constant at higher acetone contents, the position of the maximum being dependent on temperature. For about 18 wt.% acetone E and A show a maximum, whereas in solvolysis they pass through a minimum. This difference in behaviour is explained by the fact that in the latter case the transition state is more polar than the initial state and that for the reaction between ethyl bromide and hydroxyl ion the transition state is less polar than the initial state of the reactants, which means that the solvation phenomena are mainly opposite in the two cases. The isodielectric activation energies and frequency factors do not differ essentially from E and A for solvents of constant composition, whereas in the case of the uncatalysed hydrolysis they are quite different in magnitude and in behaviour.

It was found in a previous investigation [1] that very interesting details are revealed in the uncatalysed hydrolysis (solvolysis) of primary and tertiary alkyl halides when water in the solvent is progressively replaced by acetone. Investigations in this field have been continued, and the present paper reports results for the alkaline hydrolysis of ethyl bromide in acetone-water mixtures. The alkaline hydrolysis of methyl and ethyl iodides has previously been studied in methanol-water and in ethanol-water mixtures by Lobry de Bruyn and Steger [2], but at 25° only. They found that the reaction rate increases somewhat with increasing proportion of alcohol in the solvent.

EXPERIMENTAL

Chemicals. The ethyl bromide used was a commercial specimen of the highest purity. It was carefully distilled before use. The acetone was Merck's acetone *pro analysi*. The water used as the solvent in the kinetic experiments was freshly distilled.

Method. The kinetic experiments were carried out in about 12-ml ampoules of Pyrex glass. Because of the great volatility of ethyl bromide, the same method was used as in the previous investigation. Samples of 10 ml (7—9 in each run) of a 0.05 *N* sodium hydroxide solution in the appropriate solvent were measured with a pipette into the ampoules and of ethyl bromide a quantity required to form a 0.05 *M* concentration was added into each ampoule by means of an 'Agla' micrometer syringe equipped with a needle of fine bore. Blank experiments confirmed that ethyl bromide could be so measured into the solution with an accuracy of ± 0.1 mg. Ampoules were sealed and placed into the thermostat. At suitable intervals they were removed, quickly chilled by immersing into a mixture of ice and water, and opened, and the contents were washed into a slight excess of 0.1 *N* HCl-solution, pre-cooled in ice-water. The acid was titrated with 0.025 *N* sodium hydroxide solution, using cresol red as indicator. Because of the poor solubility of ethyl bromide in the highly aqueous solvents, a vigorous shaking of the ampoules before immersing into the thermostat was necessary. Shorter reaction times than one hour were not used. The error caused by the evaporation of ethyl bromide into the small gas volume (1—2 ml) over the solution in the ampoules was negligible.

CALCULATIONS

In alkaline solutions the following two reactions occur simultaneously

$$C_2H_5Br + H_2O \xrightarrow{k_1} C_2H_5OH + HBr \qquad (1)$$

$$C_2H_5Br + OH^- \xrightarrow{k_2} C_2H_5OH + Br^- \qquad (2)$$

Thus, if x is the concentration of inorganic bromide after the time t, and a is the initial concentration of ethyl bromide, and b that of sodium hydroxide, the rate equation is

$$\frac{dx}{dt} = k_2(a-x)\ (b-x) + k_1(a-x) \qquad (3)$$

For $a = b$ we have

$$\frac{dx}{dt} = k_2(a-x)^2 + k_1(a-x) \qquad (4)$$

On the basis of this equation we obtain[3] for evaluation of k_2:

$$\frac{x}{a} = \frac{1-e^{-k_1 t}}{1-\alpha e^{-k_1 t}} \qquad (5)$$

where

$$\alpha = \frac{a}{a + k_1/k_2} \qquad (6)$$

Equation (5) gives

$$\alpha = \frac{e^{-k_1 t} + \frac{x}{a} - 1}{\frac{x}{a} \cdot e^{-k_1 t}} \qquad (7)$$

If k_1 is separately determined, α can be obtained from this equation. Thus we obtain from equation (6).

$$k_2 = \frac{k_1 \alpha}{a\ (1-\alpha)} \qquad (8)$$

Table 1. The applicability of equation (8). Solvent 27.2 wt.% aqueous acetone. Temperature 60.20° C.

Time secs.	HCl 0.1012 *N* ml	NaOH 0.0248 *N* ml	$\frac{x}{a}$	$e^{-k_1 t}$	a	
3 600	4.5	1.90	0.1834	0.9712	0.8681	$k_1 = 8.12 \times 10^{-6}$ sec.$^{-1}$
5 400	4.5	3.45	0.2602	0.9572	0.8727	
7 200	4.5	4.99	0.3367	0.9434	0.8819	$a_{mean} = 0.8788$
9 000	4.0	3.93	0.3852	0.9296	0.8791	
11 100	4.0	4.85	0.4309	0.9138	0.8753	$k_2 = 117.5 \times 10^{-5}$
12 600	3.5	3.90	0.4840	0.9025	0.8848	(mole/l)$^{-1}$ sec.$^{-1}$
13 500	3.5	4.47	0.5130	0.8472	0.8912	
28 500	3.0	5.72	0.6860	0.7958	0.8826	
30 000	3.0	6.21	0.7003	0.7862	0.8836	

For the calculation, the values of k_1 were taken from the paper of Tommila, Tiilikainen, and Voipio [1].

As an illustration of the results the run given in Table 1 may be taken.

Side by side with the nucleophilic substitution there occurs an olefin-forming elimination. Under the conditions in the kinetic experiments performed in this investigation the proportion of the olefin-formation, however, is negligible [4,5] (see also Ref.[6]).

The solvolysis with water is somewhat dependent on the initial concentration of ethyl bromide; in 46 wt.% acetone at 60° C, for example, the following values were found:

Initial concentration of EtBr, moles/liter	0.2	0.1	0.05	0.01
$10^6 k_1$ sec.$^{-1}$	4.54	4.94	5.04	5.26

In Eq. (3) the variation of k_1 with respect to concentration is ignored. For the alkaline hydrolysis at the same temperature and in the same solvent it was found that for the initial concentration of the reactants 0.05 mole/l, $k_2 = 8.82 \cdot 10^{-4}$, and for 0.025 mole/l, $k_2 = 1.00 \cdot 10^{-4}$ l mole^{-1} sec^{-1}. Consequently, all the experiments referred to in the discussion were conducted with equal initial concentrations, 0.05 *M*, for both EtBr and NaOH.

All velocity constants k_2 were corrected for a change of concentration due to thermal expansion of the solvent [7]. Activation energies, *E*, and frequency factors, *A*, were obtained from the variation of log *k* with $1/T$, the method of least squares being used in the calculations. The entropy of activation, ΔS^*, was calculated by means of the equation

$$A = e \frac{kT}{h} e^{\Delta S^*/R}.$$

The results are summarized in Table 2.

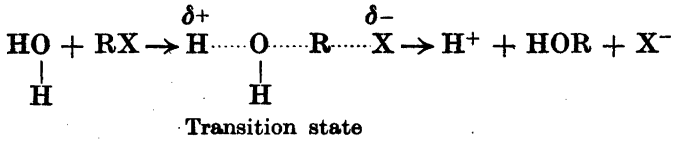
Table 2. The reaction EtBr + OH^- → EtOH + Br^- in various acetone-water mixtures.

H_2O ml/l	Me_2CO wt. %	x_{acet}	10^5 k (mole/l)$^{-1}$ sec.$^{-1}$					E cal.	log A	ΔS^* E.U.
			24.90°	39.75°	49.90°	60.20°	75.00°			
1 000	0*	0	1.59	8.00	22.7	64.4	222	20 450	10.19	−13.9
900	8.7	0.029	2.28	12.7	32.4	103	398	21 200	10.90	−10.6
800	17.8	0.063	2.63	13.7	48.6	138	529	22 000	11.50	− 7.9
700	27.2	0.104	2.83	15.6	43.3	121	482	21 100	10.91	−10.6
600	36.5	0.151	2.79	14.3	42.8	104	444	20 750	10.65	−11.8
500	46.2	0.210	2.49	12.8	35.0	91.5	347	20 300	10.30	−13.3
400	56.0	0.283	2.38	12.2	32.6	85.0	314	20 050	10.08	−14.4

* By extrapolation.

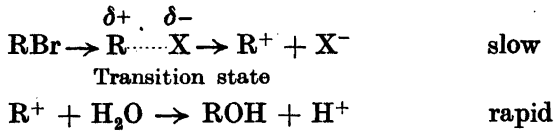
DISCUSSION

The uncatalysed hydrolysis of *n*-alkyl halides is a one-stage (S_N2) reaction



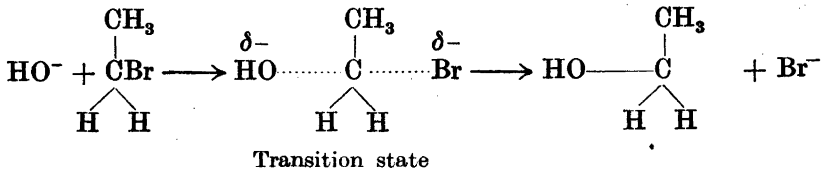
Transition state

and that of tertiary alkyl halides a two-stage (S_N1) process in which the rate-determining step is the dissociation of the alkyl halide molecule:



In both reactions the transition state involves a partial separation of electric charges and is, accordingly, more polar than the initial state. Consequently, the transition state is solvated more than the inactivated alkyl halide. The charges of the transition state tend to orientate also adjacent strongly polar acetone molecules, and thus an addition of acetone to the water increases the total solvation of the transition state, which causes a decrease in E and A. For about 20 wt. % acetone E and A show a minimum [1].

In the reaction between ethyl bromide and hydroxyl ion a distribution of the charge is involved in the transition state, which is thus less polar than the initial state of the reactants:



Transition state

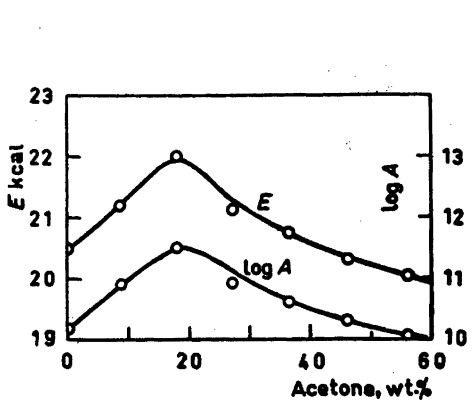


Fig. 1. Variation of E *and log* A *with the proportion of acetone in the solvent.*

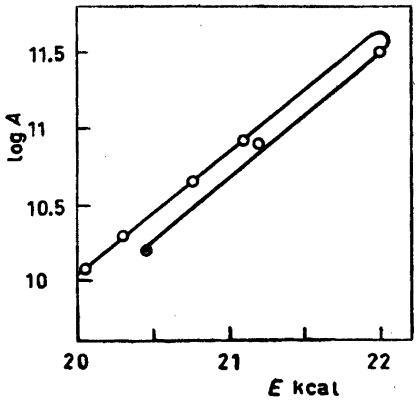


Fig. 2. Plot of log A *against* E. ⊙ *Water.*

Owing to its lower polarity the transition state is less solvated than the initial state. With increasing proportion of acetone in the solvent the total solvation of the reactants increases more than that of the less polar transition state since the electric field of the hydroxyl ion tends to organize acetone molecules more than the weaker field of the transition state. Thus E and A should increase on adding acetone to the solvent. This is in agreement with the observed solvent effect. Table 2 and Fig. 1 show that E and A are increased on increasing the proportion of acetone until the solvent contains acetone about 18 wt.%, but that after that they have a descending slope. The alkyl group of the transition state will attach acetone molecules for which they have a specific attraction and this effect increases with the proportion of acetone in the solvent, whereas the solvation of hydroxyl ions with water becomes gradually weaker. Both of these two factors tend to decrease E and A. With increasing proportion of acetone in the solvent also the solvation of ethyl bromide increases, but it is reasonable to assume that in very dilute solutions of ethyl bromide the situation is soon reached where each alkyl halide molecule has around itself a maximum number, or nearly so, of acetone molecules and that after that their number or orientation increases only little. Changes in the internal structure of the solvent may also be of consequence. It is noteworthy that the maximum lies at about the same acetone percentage as the minimum which has been observed in the case of the uncatalysed hydrolysis. The difference in polarity between the initial state of the reactants and the transition state is smaller than in the uncatalysed hydrolysis of ethyl bromide, and correspondingly the height of the maximum, with regard to the value of E in pure water (1 500 cal) is much smaller than the depth of the minimum (5 000 cal) in the latter case.

The rate constant is little affected by changes in the composition of the solvent. On adding acetone to the water, k first i n c r e a s e s, but then turns into decreasing. The position of the maximum velocity is dependent on temperature (Table 2). The influences of the changes in the activation energy and activation entropy on k are counteracting, and it is interesting that the entropy

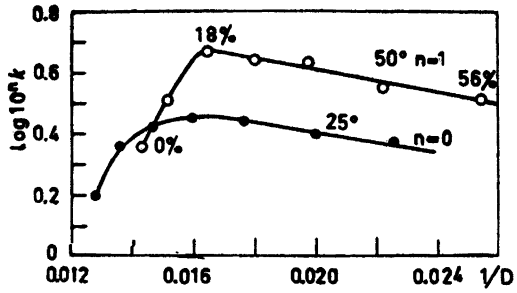


Fig. 3. Graph to show the correlation between log k *and* 1/D.

changes always dominate the rate changes. The activation energy is less than that found for the solvolysis except in the region of the maximum, but since the frequency factor is much higher than that (*i. e.* $A/[H_2O]$) for the solvolysis, the rate of the reaction between hydroxyl ion and ethyl bromide is always greater than that between water and ethyl bromide. On either side of the maximum the relationship between log A and E is linear, $\triangle \log A/\triangle E$ 0.80, *i. e.* of the same order of magnitude as for the alkaline hydrolysis of carboxylic esters between pure water and the minimum (Fig. 2).

According to Laidler and Eyring [8], for a reaction between an ion and a neutral molecule a straight line of a positive slope should be obtained for the plot of log k against $1/D$, provided the non-electrostatic effects can be neglected. The predicted change of rate with the dielectric constant [9] in the reactions between alkyl halides and hydroxyl ion seems, according to the results of de Bruyn and Steger, to exist in alcohol-water mixtures. In acetone-water, in the region from pure water to 18—27 wt.% acetone the direction of the effect is that predicted by the theory (Fig. 3), but the plot is not linear. In mixtures containing more acetone the plot is linear, but of a negative slope, which should imply that the radius of the transition state is less than that of the OH^- ion. According to Amis and Jaffé [10], for a reaction between a negative ion and a dipole an increase of the dielectric constant should cause an increase in reaction velocity.

Isodielectric solutions. The values of log k for isodielectric solutions at different temperatures were obtained from the k values of Table 2 by graphical interpolation, using for the dielectric constant, D, values given by Åkerlöf [9]. The linear relationship between log k and $1/T$ was found to hold also for iso-

Table 3. Isodielectric activation energies and frequency factors.

D	40	45	50	55	60	65
$E_D$cal.	20 500	21 000	21 500	22 000	21 650	20 450
log A_D	10.40	10.78	11.15	11.53	11.31	10.44

dielectric solutions. In the calculation of the isodielectric activation energies, E_D, and frequency factors, A_D, only temperatures of 25°, 40°, 50°, and 60° were taken into account, since for 75° the extrapolated values of the dielectric constant are very inexact. A comparison of Tables 2 and 3 shows that the isodielectric activation energies and frequency factors do not essentially differ from those for the solvents of constant composition, and what is more, they pass through a maximum. In this respect, the reaction between ethyl bromide and hydroxyl ion differs greatly from the uncatalysed water hydrolysis of alkyl halides, in which E and E_D, and A and A_D in their magnitude and behaviour differ essentially from each other.

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Oxidation of Pyridine Bases to Pyridine Carboxylic Acids with Dilute Nitric Acid at High Temperature and Pressure

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Pyridine homologues were oxidized in a stainless steel autoclave using dilute nitric acid at temperatures of about 200° C and pressures of about 30—40 kg/cm². Beta-picoline gave nicotinic acid in 50—60 % yield and gamma-picoline was oxidized to *iso*nicotinic acid in an average yield of about 93 %. Gamma-ethylpyridine also gave a very good yield (90 %) of *iso*nicotinic acid. The yield from 2,4-lutidine was 45 % and from 2,4,6-collidine 40 %.

*Iso*nicotinic acid was also prepared from mixtures of 2,4- and 2,6-lutidines as well as from commercial mixtures of higher pyridine homologues. A product of good purity was obtained in satisfactory yield. It is thus possible to produce *iso*nicotinic acid in a convenient way from cheap "by-product" pyridines.

Formerly the industrial oxidation of pyridine homologues and of quinoline and its derivatives was mainly carried out using expensive oxidizing agents such as potassium permanganate and there are still plants operating in this way. Nitric acid has been used for the oxidation of nicotine to nicotinic acid and there are several patents claiming the oxidation of different pyridine bases with nitric acid or nitrogen tetroxide in the presence of sulphuric acid at temperatures of about 300° C. Small quantities of selenium are used as catalyst [1]. Other patent specifications describe the oxidation of quinoline derivatives, *e. g.* oxiquinoline, with nitric acid in the presence of HCl[2] and methods have been given of oxidizing sulphates and phosphates of quinoline, *iso*quinoline, alkyl quinolines and picolines with nitric acid [3]. A characteristic feature of these methods is that they operate at atmospheric pressure. There are examples, however, of the process being conducted at higher pressures.

Thus, 2,5-dialkylpyridines have been oxidized with nitric acid of 60 % concentration at 160—200° C under pressure to give pyridine-2,5-dicarboxylic acid [4]. Using such comparatively concentrated nitric acid under pressure can be expected to cause explosions and such have been reported. Oxidation

of 2-methyl-5-ethylpyridine with air under pressure in the presence of HNO_3 was recently reported in a patent [5], and the oxidation of this compound and beta-picoline with nitric acid at 150—180° C in the presence of catalysts was recently claimed [6]. The oxidation of 2-methyl-5-ethylpyridine with dilute nitric acid under pressure was originally carried out by Krzikalla [7] and nicotinic acid is now made on an industrial scale at 185° C and about 20 atm. pressure [8]. No details are, however, given as to the method of carrying out this process and nothing has been published concerning the possibility of using high pressure oxidation with dilute nitric acid for the industrial manufacture of *iso*nicotinic acid and nicotinic acid from other pyridine bases. This paper gives a concentrated survey of experimental work which had the purpose of studying technical methods of making nicotinic acid and *iso*nicotinic acid out of various raw materials by high pressure oxidation with dilute nitric acid.

Nicotinic acid can be made from beta-picoline in fairly good yield by oxidizing with 10 % HNO_3 at 230° C and 35—40 atm. Higher yield but lower conversion was obtained at 200° C.

*Iso*nicotinic acid was made from various raw materials. Gamma-picoline gave a 93.5 % yield with HNO_3 of 10 % concentration at 230° C and 40 atm. and gamma-ethylpyridine was oxidized at 200° C and 30—40 atm. in a 90 % yield. 2,4-Lutidine gave a 45 % yield and 2,4,6-collidine gave a 40 % yield under similar conditions.

It is however rather expensive to use these pure compounds as raw materials for the production of *iso*nicotinic acid and, also they have sometimes been in very short supply. Therefore a method has been developed, which uses low-priced mixtures of pyridine bases instead of pure isomers. Such mixtures have been oxidized with dilute nitric acid at about 200° C and 30—40 atm. to give *iso*nicotinic acid of good purity in satisfactory yield. This makes it possible to produce this new pharmaceutical intermediate at low cost using readily available "by-product" pyridines.

Most of the processes reported in this paper have also been run successfully on a semi-technical scale. The technical performance of the methods towards semi-continuous and continuous methods has also been studied.

EXPERIMENTAL

The oxidations were carried out in a stainless steel autoclave according to a procedure described in a previous paper [9]. Some experiments were run in the presence of phosphoric acid which was shown to prevent corrosion and sometimes also to improve the yield.

Oxidation of beta-picoline. β-Picoline (50 g) was oxidized with an equivalent quantity of 10 % nitric acid in the presence of 60 g of 89 % orthophosphoric acid at 230° and 35—40 atm. pressure. The nitric acid was added in 2 portions and the total reaction time was 2 hours. The volume of the autoclave was 2.5 litres. Since a homogeneous solution was formed no stirring was necessary.

The autoclave was then discharged through a riser by means of the pressure in the autoclave. By evaporation of the reaction product, removing the rest of the nitric acid by heating with ethyl alcohol and esterification with ethyl alcohol, the nicotinic acid formed was converted to the ethyl ester. In a series of experiments this was obtained in a 50—60 % yield (calculated on converted picoline) and had a satisfactory degree of purity. (Saponification value 365—376). About 40 % of the picoline was unchanged and

was recovered. The corrosion of stainless steel proved to be as low as 0.1 – 0.2 g per m^2 per hour.

The process can also be conducted continuously, by pumping in the reactants using high pressure pumps and discharging the contents of the autoclave continuously through a riser. The acid can also be added portionwise (all the organic compound being added at the start of the reaction) so that excess of acid is present only towards the end of the reaction.

In another series of experiments the oxidation was carried out as above but the nicotinic acid was obtained by evaporation of the acid solution and adjusting the pH value to 3.5. A further amount of nicotinic acid could be obtained *via* the copper salt (unconverted picoline being removed by distillation), after raising the pH value.

When the picoline was oxidized as above but at 200° C, the reaction was slower, necessitating a longer reaction time, but the yield was higher.

β-Picoline was also oxidized in the same way without the addition of phosphoric acid The yield was somewhat lower and the corrosion was more serious.

Oxidation of gamma-picoline. Gamma-picoline (90 g), an equivalent quantity of 10 % nitric acid and 89 % orthophosphoric acid (108 g) were heated in an autoclave at 230° C and a pressure of 40 kg per cm^2. The reaction was allowed to proceed for one hour after the temperature had reached 230° C. While still warm the batch was discharged and concentrated to one-half the original volume.

After cooling, part of the product precipitated. After adjusting the pH value to 3.5, the *iso*nicotinic acid was filtered off and washed with water. To the mother liquor, which contained a small amount of *iso*nicotinic acid, alkali was added until the pH value was 7, and unconverted picoline was then distilled with steam.

The steam distillation was discontinued when the distillate no longer smelt of picoline and in this way approximately 15 % of the original amount of picoline was recovered. The picoline solution was used for a new run. After having precipitated the phosphoric acid with $CaCl_2$, the *iso*nicotinic acid remaining in the mother liquor was precipitated as the copper salt. After boiling it with NaOH, *iso*nicotinic acid could be obtained.

In a series of such oxidations the average yield was 93.5 %. The equivalent weights of the crude product varied from 122.4 to 123.3 against the theoretical 123.1. The tertiary nitrogen content was in very good agreement. The crude product could be converted directly to pure *iso*nicotinic acid hydrazide, in high yield.

Other experiments were carried out as above, except that initially a smaller quantity of the components was run in and then a solution of gamma-picoline in nitric acid and phosphoric acid was gradually added in portions as each preceding addition had reacted. For the first charging a 10 % acid was used, and then 30 % nitric acid. In this way a higher capacity was attained. The portionwise addition can also be accomplished by means of high-pressure pumps, and the procedure can be made continuous by continuous discharge *via* a riser.

Oxidations were also carried out without the addition of phosphoric acid. The yield was of the same order of magnitude but the corrosive attack was more severe.

Oxidation of gamma-ethylpyridine. Gamma-ethylpyridine was oxidized with 30 % HNO_3, in 10 % excess, at 180 – 200° C and 30 – 40 kg per cm^2. The reaction time was one hour after the temperature had reached 180° C. The main part of the *iso*nicotinic acid was obtained on adjusting the pH value to 3.5 and a further small quantity was isolated *via* the copper salt. The yield was about 90 %. Oxidations were also carried out in the same way with 10 % HNO_3 and in the presence of phosphoric acid. The yield was about the same.

In preparing gamma-ethylpyridine by treating pyridine with acetic anhydride and zinc there was obtained, after the ethyl pyridine fraction had been steam-distilled, a fraction boiling at 175 – 210° C (760 mm Hg). This was oxidized with HNO_3 as above and *iso*nicotinic acid in 60 % yield was obtained.

Isonicotinic acid from 2,4-lutidine and 2,4,6-collidine. 2,4-Lutidine, technical grade, was oxidized with 20 % HNO_3, in equivalent amount in the presence of phosphoric acid. The temperature was 230° C, the pressure 40 kg per cm^2 and the reaction time at 230° C one hour. *Iso*nicotinic acid was then obtained in 45 % yield, not taking into account the unconverted lutidine. The product was obtained partly by direct precipitation at pH 3 and partly *via* the copper salt.

45 g of 2,4,6-collidine and 30 % nitric acid in 10 % excess were placed in a laboratory autoclave together with phosphoric acid and heated without stirring to about 180° C when the reaction started. This first batch having reacted, a total amount of 90 g of collidine and a corresponding quantity of 30 % acid in 10 % excess were fed into the autoclave in several portions. The temperature was maintained at 190—200° C and the pressure at 30—40 kg per cm² for the whole period. Nitrogen oxides were expelled from time to time. After the last addition, the autoclave was allowed to stand at 200° C and 40 kg pressure for one hour.

The acid solution was evaporated to give, on cooling, 21 g of *iso*nicotinic acid. After further evaporation and adjustment of the pH value to 3.5, an additional 34 g were isolated. Thereafter 15 g of copper *iso*nicotinate and a portion of unconverted collidine were obtained.

Isonicotinic acid from mixtures of pyridine bases. 80 g of a synthetic mixture of *ca.* 42 % gamma-picoline, 34 % beta-picoline and 24 % 2,6-lutidine were placed in an autoclave together with 1 275 g of 10 % HNO_3, and the batch was heated to 190° C. The temperature rose to a maximum of 235° C, and the pressure to a maximum of 40 kg per cm². The reaction time at 220—230° C and 30—40 kg per cm² was approximately one hour. The reaction mixture was then evaporated to a volume of about 450 ml at which point 25 g of a product precipitated which, judging by the melting point and equivalent weight, consisted of *iso*nicotinic acid. Without being subjected to any purification process, this crude product was used for preparing the hydrazide. The compound obtained was, according to the analyses, pure *iso*nicotinic hydrazide. The yield from the hydrazide synthesis was quite as good as when pure *iso*nicotinic acid was used as the raw material. From the mother liquor from the oxidation an additional amount of *iso*nicotinic acid could be obtained by precipitation of the copper salt.

In the same way 45 g of a commercial mixture of pyridine bases with a boiling range of 164—181° C at 760 mm Hg were oxidized with 10 % HNO_3 in 10 % excess (the mixture being considered as collidine) at 200—210° C and 35—40 kg per cm². After evaporation, 10 g of a product was obtained having an equivalent weight of 124.5. This product was converted, without further purification, to the hydrazide in the usual way and a product was obtained which, on the basis of the analyses, was pure *iso*nicotinic hydrazide. An additional amount of *iso*nicotinic acid could be obtained from the mother liquor.

Other experiments were run as above except that the autoclave was charged with the mixture of pyridine homologues and 30 % nitric acid in 10 % excess. When this batch had reacted, a further amount of the homogenous mixture in 40 % HNO_3 was successively run in *via* pressure cylinders. The reaction took place at 200—210° C, and 40 kg per cm². The reaction time, after all the reactants had been added, was one hour at the temperature and pressure stated.

When the reaction was completed, the batch was discharged while boiling off the water, and the pH value was adjusted to 3.5 without further evaporation. After cooling, *iso*nicotinic acid precipitated and this was converted, without purification, to the pure hydrazide in good yield. In applying this procedure, the capacity of the apparatus turned out to be very high. In a 2.5 litre autoclave approximately 200 g of pyridine homologues could be oxidized per batch. The yield in such tests has been 40 g of *iso*nicotinic acid per 100 g of mixture of pyridine homologues. A stronger acid, *e. g.* 50 %, can also be added to the autoclave, and the oxidation can be carried out continuously in the autoclave as well as in tubes.

Further oxidations were run as follows:

Into an autoclave was pumped a solution of 4.5 parts by weight of the homogenous mixture, just referred to, in an amount of 40 % nitric acid corresponding to 10 % excess, the mixture of pyridine homologues being calculated as collidine. Meanwhile the autoclave was heated to 180—190° C. The reaction started when the temperature had reached this value and proceeded at 190—195° C and 30 kg per cm² for 2 hours. The autoclave was filled to 70 % of its volume. The solution was blown out through a riser and evaporated in vacuum to approximately one third of its original volume. After adjusting the pH value to 3.5, and centrifuging, washing and drying, 1.5 parts by weight of a yellow product were obtained. By recrystallizing from water a white product was obtained which, on analysis, proved to be pure *iso*nicotinic acid. The yield on recrystallization was over 90 %. The crude *iso*nicotinic acid was used directly and with excellent results for preparation of the hydrazide.

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Thermochemical Investigations on Organic Sulfur Compounds

IV. On Thermochemical Sulfur Bond Energy Terms

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From modern values of heats of combustion of sulfur compounds the different sulfur bond energies have been computed. Assuming the heat of sublimation of graphite and sulfur to be 138 and 66.3 kcal.gatom^{-1}, respectively, the following values have been obtained: E_{C-S} = 61.5 kcal., $E_{C=S}$ = 115 kcal., E_{S-H} = 87.5 kcal. and E_{S-S} = 67 kcal.

The following heats of formation at 20° C have been computed from combustion experiments: 5-Thianonane $\Delta Hf°$ (liq) = −52.7 ± 0.45 kcal. mole^{-1}; Methyl *iso*thiocyanate $\Delta Hf°$ (s) = 18.7 ± 0.30 kcal. mole^{-1} and 1-Pentanethiol $\Delta Hf°$ (liq) = −36.2 ± 0.20 kcal.mole^{-1}.

For many problems in organic thermochemistry it is very useful — and sometimes necessary — to have reliable bond energy data available. Recently, Cottrell made a careful compilation of bond dissociation as well as thermochemical bond energy data [1]. The given values for sulfur bond energies originate partly from old determinations of heats of combustion. It therefore seemed desirable to check these values. Besides, it was judged possible to increase the constancy of obtained data for the same bond by comparing pairs of compounds with almost the same intramolecular environment and then relate the energy of the differing bonds to each other*. The application of this method is often successful — the calculation of the S—S bond energy may illustrate this approach.

THE C—S BOND ENERGY

Reliable values for heats of formation of dialkylsulfides are available for 3-thiapentane [2] and 5-thianonane. A comparison between the thiaalkane and the corresponding normal alkane gives the heat of substitution of a CH_2-group for a sulfur atom (Table 1).

* *Cf.* the group equivalent method, see Refs.[4,5].

$$Alk_2S\ (g) + C\ (graphite) + H_2\ (g) \rightarrow Alk_2CH_2 + S\ (rhombic) + \Delta Hr \quad (I)$$

Table 1. Comparison between heats of formation of thiaalkanes and alkanes.

Alk.	ΔHf kcal.mole^{-1}		ΔHr kcal.
	Alk_2S	Alk_2CH_2	
Et	−19.9	−35.00 [3]	−15.1
Bu	−40.3	−54.75 [3]	−14.4
$Alk_2 = (CH_2)_5$	−15.14 *	−29.43 [3]	−14.3

* The average between the value −14.96 ± 0.25 kcal. obtained at the US Bur. Mines, Bartlesville [6] and our value −15.32 ± 0.20 kcal.[7].

From ΔHr, the C—S thermochemical bond energy, E_{CS}, is calculated in terms of E_{CC}, the heat of atomization of sulfur, ΔHa(S), and the well known increment in the heat of formation per CH_2-group (4.926 kcal.) [8].

$$E_{CS} = \tfrac{1}{2}\ (E_{CC} + \Delta Ha(S) + \Delta Hr + 4.926) \quad (1)$$

Using the values $E_{CC} = 66.2$ kcal.[1] and $\Delta Ha(S) = 66.3$ kcal.[1] we get $E_{CS} = 61.2$ and 61.5 kcal. for Et_2S and Bu_2S, respectively.

An estimate of E_{CS} may also be obtained in the same way from the known heat of formation of thia*cyclo*hexane, assuming the ring to be strainless. The calculation leads to $E_{CS} = 61.6$ kcal. Within the expected errors the three values are identical. We therefore use the average and put

$$E_{CS} = 61.5 \text{ kcal.}$$

This value is primarily valid for straight chain dialkyl and unstrained ring sulfides but will also be used for the calculation of the thermochemical sulfur-hydrogen and sulfur-sulfur bond energies.

THE C=S BOND ENERGY

A direct determination of the C=S bond energy is at present not possible due to the fact that thiones without electron-interfering neighbors are not stable as monomers. The few exceptions known have no unambiguous structure; *cyclo*hexanthione exists in two protomeric forms, *viz. cyclo*hexanthione and 1-mercapto-*cyclo*hexen [9]. In compounds available for an experimental investigation the heat of formation of a C=S bond is made up of two quantities, the (by definition) invariable C=S bond energy and the energy supplied by interaction between the C=S group and its environment **. However, it is

** As in the case of the C=O bond, the C=S bond energy implicitly includes the stabilizing energy from the interaction between the C=S and $\overset{+}{C}—\overset{-}{S}$ structures.

possible to give a relation between the bond energy and the interaction energy (resonance energy) for resonating molecules containing a C=S bond.

Pauling estimated the C=S bond energy to be 103 kcal. and the resonance energy for CS_2 to be 11 kcal., for COS 20 kcal.; for CO_2 the resonance energy is 33 kcal.[10]. Using data for CS_2 and COS from *Selected Values* [11], the heats of formation of monoatomic gaseous carbon [10] and sulfur [10] and the estimated C=S bond energy 103 kcal., the resonance energy for CS_2 becomes 23 kcal. per mole, and for COS 28 kcal. per mole. Either the C=S bond energy or the resonance energies for COS and CS_2 must be in error. If the resonance energies given by Pauling are correct, a C=S bond energy of about 112 kcal. should be expected.

From bond distances (see Pauling) and from infrared measurements [12] it is implied that the character of the resonance in CO_2, COS and CS_2 is the same, the three structures $X{=}C{=}Y$, $\bar{X}{-}C{\equiv}\overset{+}{Y}$, $\overset{+}{X}{\equiv}C{-}\bar{Y}$ contributing equally (X, Y = O, S). Under these circumstances the decrease in interaction energy from CO_2 over COS to CS_2 is in accordance with the weaker C—S bonds compared with the C—O bonds. It thus seems justified to use the values of the resonance energies given by Pauling as fairly good approximations.

The endothermicity of the reaction

$$\text{C (g)} + 2\ \text{S (g)} = CS_2\ \text{(g) may be expressed as}$$
$$\Delta Hf\ \text{(atomic)} = -2E_{C=S} - R_{CS_2} \qquad (2)$$

where $E_{C=S}$ is the energy of the C=S bond and R_{CS_2} the resonance energy of CS_2.

From the heat of formation of gaseous CS_2[11] and gaseous monoatomic carbon [1] and sulfur [1]:

$$E_{C=S} = 121 - 0.5\ R_{CS_2}\ \text{kcal.} \qquad (3)$$

Using $R_{CS_2} = 11$ kcal., $E_{C=S} = 116$ kcal.
Analogously, for COS (using $E_{C=O} = 162$ kcal.[1]):

$$E_{C=S} = 134 - R_{COS} \qquad (4)$$

$R_{COS} = 20$ kcal. gives $E_{C=S} = 114$ kcal.

It should be possible to determine the C=S bond energy more precisely from the heat of combustion of a compound with a small resonance energy. The resonance energy of the alkyl *iso*cyanates, according to Pauling, is 7 kcal. and for the thiocyanates it should be less than that. We therefore redetermined the heat of combustion of methyl*iso*thiocyanate and found

$$\Delta Hf = 27.1\ \text{kcal. per mole}$$

in good agreement with Thomsen's value of 28 kcal. Using Cottrell's bond energy values, we found

$$E_{C=S} + R_{MeNCS} = 118\ \text{kcal.} \qquad (5)$$

If we put $R_{MeNCS} = 3$ kcal., we get $E_{C=S} = 115$ kcal.

The somewhat arbitrary choice of resonance energy values gets support from equations (3) to (5). These equations combine to give:

$$R_{COS} = 0.5\ R_{CS_2} + 13 \quad (6)$$

$$R_{COS} = R_{MeNCS} + 16 \quad (7)$$

R_{MeNCS} is of course positive but should be less than 7 kcal., a probable value is 3—5 kcal. Thus, $23 > R_{COS} > 16$, it is believed that 19—21 kcal. should be a fair estimate. The limits for the R_{CS_2} value will then be $20 > R_{CS_2} > 6$, and probably $16 > R_{CS_2} > 12$. The apparently proportional decrease in interaction energy in the series, CO_2, COS and CS_2 favors the low limit value of $R_{COS} = 19$, which gives $R_{CS_2} = 12$, $R_{MeNCS} = 3$ and $E_{C=S} = 115$ kcal. We will therefore use this value as a fairly good estimation of the C=S bond energy.

$$E_{C=S} = \mathbf{115\ kcal.}$$

Pauling and Sherman deduced the resonance energy of MeNCS to be 10 kcal. From this value, according to equation (3)—(5), $R_{COS} = 26$ kcal. and thus $R_{CS_2} = 26$ kcal. which is not very probable. It is worth noticing that equations (3) to (5) are based only on

1) the assumption that it is possible to express the energy of a resonating molecule as a sum of constant bond energies plus a resonance energy term (which in fact only indicates the deviation from the constancy of the bond energies);

2) experimental data of heats of combustion;

3) the values of bond energies given by Cottrell; concerning the $E_{C=N}$-term value, which differs appreciably from previous values in the literature, reference is made to the further discussion.

These assumptions being essentially correct, the equations (3) to (5) relate the resonance energy terms for different compounds to each other. The conclusion as to the probable value of the termochemical C=S bond energy is drawn from the fair assumption that the interaction energy changes in a regular manner in the series CO_2, COS and CS_2.

Cottrell prefers to incorporate the resonance energy term into the value of the thermochemical bond energy and gets $E_{C=S} = 121$ kcal. both for COS and CS_2. This agreement is reached by using the value $E_{C=O} = 175$ kcal. which is calculated for CO_2 and which also includes the resonance energy term. However, from Cottrell's data, the calculated atomic heat of formation of MeNCS exceeds the experimentally determined value by 3 kcal.; for MeNCO the calculated value exceeds the experimental by as much as 22 kcal. if $E_{C=O} = 175$ kcal. is used. Excluding the resonance energy from the bond energy term and using $E_{C=O} = 162$ kcal. (Ketones; Cottrell) and $E_{C=S} = 115$ kcal. (this paper) the resonance energy of MeNCS becomes 3 kcal. (assumed value) and the calculated atomic heat of formation of MeNCO still exceeds the experimental value by 9 kcal. The heat of combustion of methyl *iso*cyanate was determined by Lemoult in 1898 [13] and might be seriously in error. This suspicion is strengthened by the fact that Pauling [10], Glockler [14], Syrkin and Dyatkina [15] and others have used the heat of formation of MeNCO to calculate the C=N bond energy and give values between 90 and 94 kcal. while Cottrell, who has used a deter-

mination of the heat of formation of *n*-butyl *iso*butylidine amine from Coates and Sutton [16] obtains the much higher value of 112 kcal. (Recalculated from Pauling's values for heats of atomization of carbon and nitrogen it becomes 105 kcal.).

A comparison between the heats of formation of the following pairs of compounds also indicates an abnormal situation for the pair methyl *iso*thiocyanate and methyl *iso*cyanate (Table 2).

$$R{=}S\ (g) + O\ (g) \rightarrow R{=}O\ (g) + S\ (g) + \Delta Hr \qquad (II)$$

Table 2.

R =	ΔHr kcal.
OC=	−54.1
SC=	−53.3
MeNC=	−34.7
$(NH_2)_2C$=	−50.5

THE S—H BOND ENERGY

The S—H bond energy was calculated from the known heats of formation of 1-pentanethiol, 2-propanethiol and hydrogen sulfide. From the heat of formation of H_2S Cottrell calculates $E_{SH} = 87.7$ kcal.[1]. In order to obtain an E_{SH} value for a normal mercaptane we determined the heat of combustion of 1-pentanethiol and found the heat of formation of the gaseous substance to be $\Delta Hf = -26.7$ kcal.*. A comparison between 1-pentanethiol and *n*-pentane gives the desired value of E_{S-H}

$$C_5H_{11}{-}H + S\ (g) \rightarrow C_5H_{11}{-}S{-}H\ \ \Delta H = -58.9\ \text{kcal.} \qquad (III)$$

$$E_{CH} - E_{CS} - E_{SH} = -58.0 \qquad (7)$$

$E_{CH} = 90.5$ kcal.[1] and $E_{CS} = 61.5$ gives $E_{SH} = 87.0$ kcal. Recently, the Bartlesville group has determined the heat of formation of 2-propanethiol [18]. From their result one gets $E_{SH} = 87.5$ kcal. Thus a value of 87.5 kcal. for E_{S-H} can be adopted with fair confidence.

$$\mathbf{E_{S-H} = 87.5\ kcal.}$$

THE S—S BOND ENERGY

High precision determinations of heats of combustion of disulfides are lacking in the literature. However, Franklin and Lumpkin [19] burned the dimethyl- and diethyl disulfides in a static bomb system. Their result for 2-methyl-2-propanethiol shows a fairly good agreement with the value from the US Bureau of Mines group [17] and the results obtained seem to be accurate within ± 1 kcal.

* The average has been used between the values obtained at the US Bureau of Mines, Bartlesville, $\Delta Hf°$ (liq) = −36.12 kcal.[17] and in this laboratory, $\Delta Hf°$ (liq) = −36.23 kcal.

The S—S bond energy may be obtained from a comparison between the heats of formation of 3-thiapentane and diethyldisulfide, ΔHf (g) = —21.4 kcal.*.

Et — S — Et (g) + S (g) → Et — S—S — Et (g); ΔH = —67.8 kcal. (IV)

which gives E_{SS} = 67.8 kcal.

In the same manner one gets from dimethyldisulfide, ΔHf = —7.2 kcal., and dimethylsulfide, ΔHf = —6.9 kcal.[11], E_{SS} = 66.6 kcal. **.

From the heats of formation of liquid H_2S_2 [11] and H_2S [11] and an estimation of the heat of vaporization of H_2S_2 using Klages equation [4] (ΔH_{vap} = 8.0 kcal.), E_{SS} = 59 kcal.. However, the calculated value of the heat of formation of H_2S_2 can be appreciably in error and therefore no particular emphasis can be put on this E_{SS}-value.

Pauling has used the heat of atomization of rhombic sulfur to calculate E_{SS} [10]. ΔHa (S) = 66.3 kcal. is combined with the heat of vaporization for S(rh) → S_8 (g), 24.35 kcal.[21], which gives E_{SS} = 63.3 kcal. However, this result is believed to be only of limited value for disulfides, considering the increased opportunity for intramolecular interaction in the S_8-ring as compared with that in a normal disulfide. Therefore, for organic disulfides the value

$$E_{SS} = 67 \text{ kcal.}$$

will be adopted.

HEATS OF COMBUSTION ***

Materials. The samples of 5-thianonane and methyl *iso*thiocyanate were prepared and purified at this laboratory. The thianonane was repeatedly distilled at different pressures, all fractions during the last distillation boiled at the same constant temperature, b.p.$_{25}$ 95.10 ± 0.05°, b.p.$_{71}$ 112.20 ± 0.05°, n_D 1.4572. The methyl *iso*thiocyanate was purified after distillation by repeated sublimations to constant m. p.

The pentanethiol was obtained from the U.S. Bureau of Mines, Thermodynamics Laboratory, Bartlesville, U.S.A., as "Samples of API—BM certified sulfur compounds purified at the Laramie Station of the Bureau of Mines and made available by American Petroleum Institute Research Project 48 A on the Production, Isolation and Purification of Sulfur Compounds and the Measurement of their Properties"****.

Apparatus and method. The calorimeter and accessory apparatus, experimental procedure and method of calculation have been described in detail previously [7,22].

Units of measurement and auxiliary quantities. The results of the combustion experiments are expressed in terms of the defined calorie equal to 4.1840 abs. joules and refer to the isothermal process at 20° and to the true mass. The molecular weights were computed from the 1951 table of international atomic weights. The observed values for the heat of the bomb process were corrected to obtain values for the energy of the idealized combustion reaction in which all the reactants and products are in their standard states at 20° and no external work is performed. The corrections, which include those from the formation of nitric and nitrous acids and for the dilution of sulfuric acid were made as described by Sunner [7,22]. The heats of formation of water and carbon dioxide at 20° were taken to be 68.355 and 94.050 cal.g^{-1}, respectively.

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* Franklin and Lumpkin give the value —20.0. From Scott *et al.* [20] the heat of vaporization is deduced to be 11.1 kcal. which gives ΔHf (g) = —21.4 kcal.

** Cottrell deduces E_{SS} = 70 and 67 kcal. for diethyl- and dimethyldisulfide, respectively.

*** Together with B. Lundin.

**** We are indebted to Dr. Guy Waddington and Mr. Ward Hubbard of the Thermodynamics Laboratory, U. S. Bureau of Mines, Bartlesville, for furnishing this sample.

Energy equivalent of the calorimeter. The energy equivalent of the calorimeter has been given elsewhere [22] as $S_B = 5251.3 \pm 0.4$ cal. per I.U. (= interval unit, equals 1.35° C). A minor change in the system gave $S_B = 5255.1$ cal. per I.U.

Heat of combustion of paraffin oil. Paraffin oil has been used as an auxiliary substance. Table 3 gives the results of two series of determinations of the heat of combustion of the oil.

Table 3. Heat of combustion of paraffin oil. The head lists S_B the energy equivalent of the calorimeter excluding the charge of substance (s), which is included in S_F; q_{CO_2} the heat of solution of carbon dioxide and q_{WC} the Washburn corrections. The columns list the amount of oil burned, the corrected increase in temperature in interval units, the heat of combustion of the fuse, the correction for the formation of nitrogen acids and the heat of combustion of the paraffin oil under standard conditions.

Oil mg mass	ΔT_{corr} I.U.	q_{fuse} cal.	q_N cal.	$-\Delta E^0c/M$ cal.per g mass.
$S_B = 5251.3$ cal. per I.U., $S_{F_2} = 0.8$ cal. per I.U., $q_{CO_2} = 5.1$ cal., $q_{WC} = 0.8$ cal.				
474.52	1.00216	14.7	9.4	11027.1
471.55	0.99616	17.3	9.6	11025.5
471.61	0.99600	15.2	9.6	11026.9
471.05	0.99450	14.0	9.6	11025.8
470.98	0.99483	15.8	9.4	11025.9
			Average	11026.2
$S_B = 5255.1$ cal. per I.U.				
471.08	0.99441	15.3	9.4	11027.8
469.27	0.99010	14.2	9.7	11025.6
472.70	0.99754	14.9	9.5	11027.1
471.75	0.99507	14.3	9.6	11024.3
473.03	1.01869	12.6	9.7	11025.8
473.22	0.99847	13.7	9.4	11026.5
			Average	11026.2

Average for both series $-\Delta E^0c/M = 11026.2$ cal. per g mass.

Correction for the heat of formation of sulfuric acid. The application of the Washburn corrections to the combustion of a compound containing sulfur means that the heat of combustion has been calculated for an idealized process, the final state of which is made up of CO_2 (g, 1 atm) and a diluted sulfuric acid, H_2SO_4, n H_2O; n usually being about 115.

In order to minimize the influence from possible systematic errors due to the presence of sulfur, the heat of combustion of rhombic sulfur has been determined under strictly comparable conditions and the final reaction has been calculated for the formation of rhombic sulfur:

$$C_aH_bS_d \text{ (s or liq)} + \left(a + \frac{b}{4} + \frac{3d}{2}\right) O_2 \text{ (g)} + \text{aq} =$$
$$aCO_2 \text{ (g)} + dH_2SO_4, nH_2O - \Delta E^0c' \qquad \text{(V)}$$

$$S \text{ (rhomb)} + \frac{3}{2} O_2 \text{ (g)} + \text{aq} = H_2SO_4, nH_2O - \Delta E^0c'' \qquad \text{(VI)}$$

Substracting d times VI from V gives:

$$C_aH_bS_d \text{ (s or liq)} + \left(a + \frac{b}{4}\right) O_2 \text{ (g)} =$$
$$aCO_2 \text{ (g)} + \frac{b}{2} H_2O \text{ (liq)} + d \text{ S (rhomb)} - \Delta E^0c' + d\Delta E^0c'' \qquad \text{(VII)}$$

The value of $\Delta E^0c''$ for n = 115 is given in Table 4 for four different series of experiments.

Table 4.

Sample	Investigator	Amount of sulfur milliatoms	$-\Delta Ec^{\circ}$ (115) cal. per milliatom of sulfur
1	A	5	143.05 ± 0.05
1	B	5	143.05 ± 0.05
1	B	8	143.10 ± 0.10
2	B	5	143.20 ± 0.10

Average for all determinations 143.10 ± 0.05 cal. per millimole of H_2SO_4, 115 H_2O *.
Deviations from a final dilution of H_2SO_4, 115 H_2O are accounted for by using SVCTP values of heats of formation of sulfuric acid at different dilutions [11].

RESULTS

The results of the individual combustion experiments are presented in Table 5.

Table 5. Results of combustion experiments. S_B = *5251.3 cal. per I.U., 5255.1 cal. (x)*

Sample mg mass	Oil mg mass	ΔT_{corr} I.U.	q_{fuse} cal.	q_N cal.	$-\Delta E^{\circ}c/M$ cal. per g mass.
		5-Thianonane.			
M = 146.290, D_4^{20} = 0.840, S_F = 0.6 cal., q_{CO_2} = 4.7 cal. q_{Wc} = −0.4 cal.					
354.17	151.81	0.99528	15.4	10.2	8967.4
334.24	171.60	0.99947	16.1	10.7	8969.9
343.71	161.02	0.99498	15.4	10.7	8968.9
350.36	157.44	1.00053	16.2	10.8	8973.8
371.50 x	137.21	0.99722	15.6	10.7	8972.8
				Average	8970.6
		Methyl *iso*thiocyanate.			
M = 73.118, D_4^{20} = (1.1), S_F = 0.5 cal., q_{CO_2} = 4.9, 5.0, 4.6 cal., q_{Wc} = 0.0 cal.					
188.36	355.52	0.974528	14.9	12.5	4223.2
341.23	247.71	0.985021	14.0	13.6	4229.8
237.57	315.75	0.949528	16.7	14.0	4227.6
				Average	4227
		1-Pentanethiol.			
M = 104.212, D_4^{20} = 0.85, S_F = 0.6 cal., q_{CO_2} = 4.7 cal., q_{Wc} = −0.4 cal.					
388.86	138.53	0.99671	13.8	10.9	8084.8
403.96	129.46	1.00509	15.1	11.0	8084.3
413.60 x	120.61	1.00282	15.0	10.0	8082.8
364.54	157.83	0.99365	15.5	10.4	8084.6
				Average	8084.1

* This value is 0.3 kcal. higher than that obtained at the US Bureau of Mines, Bartlesville.

From the results the following values of ΔHf (liq) and ΔHf (g) have been computed at 20° C.:

$$8C \text{ (graphite)} + 9H_2 \text{ (g)} + S \text{ (rhombic)} \rightarrow$$
$$C_8H_{18}S \text{ (liq)}; \Delta Hf^\circ \text{ (liq)} = -52.7 \pm 0.45 * \text{ kcal. mole}^{-1} \quad \text{(VIII)}$$

The heat of vaporization was estimated from boiling point *versus* pressure data. Stull's compilation [23] of vapor pressures gives 12.8 kcal. mole^{-1}; our own data 12.0 kcal. mole^{-1}. The average value, 12.4 kcal. mole^{-1}, has been used. For

$$2C \text{ (graphite)} + 1\tfrac{1}{2}H_2 \text{ (g)} + \tfrac{1}{2}N_2 \text{ (g)} + S \text{ (rh)} \rightarrow$$
$$CH_3NCS \text{ (s)}; \Delta Hf^\circ \text{ (s)} = +18.7 \pm 0.30 * \text{ kcal. mole}^{-1}. \quad \text{(IX)}$$

The heat of vaporization was calculated from vapor pressure data [24] to + 8.4 kcal. mole^{-1}. Thus, ΔHf (g) = + 27.1 kcal. For

$$5C \text{ (graphite)} + 6H_2 \text{ (g)} + S \text{ (rhombic)} \rightarrow$$
$$C_5H_{11}SH \text{ (liq)}; \Delta Hf^\circ \text{ (liq)} = -36.2 \pm 0.20 * \text{ kcal. mole}^{-1} \quad \text{(X)}$$

ΔH_{vap} was estimated from vapor pressure data to 9.5 kcal. mole^{-1}. Thus ΔHf (g) = —26.7 kcal. mole^{-1}.

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Thermochemical Investigations on Organic Sulfur Compounds

V. On the Resonance Energy of Thiolacetic Acid, Thiourea, Thiosemicarbazide, Thiophene and Thianthrene

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From determinations of heats of combustion and from values of thermochemical bond energies given in communication IV [1], the following resonance energy values have been computed: thiolacetic acid 4—5 kcal. mole^{-1}; thiourea 27 kcal. mole^{-1}; thiosemicarbazide 28 kcal. mole^{-1}; thiophene 20 kcal. mole^{-1} and thianthrene (resonance energy in excess of that in the two benzene nuclei) 17 kcal. mole^{-1}.

The following heats of formation have been computed from combustion experiments: Thiophene $\Delta Hf°$ (liq) = 19.13 ± 0.15 kcal. mole^{-1} at 25° C and Thiolacetic acid $\Delta Hf°$ (liq) = −51.5 ± 0.35 kcal. mole^{-1} at 20° C.

Thiolacetic acid. A comparison between the heats of formation of gaseous thiolacetic acid, ΔHf (g) = —42.6 kcal. mole^{-1}, and gaseous monomeric acetic acid, ΔHf (g) = —103.9 [2,3], may be used to calculate the difference in resonance energy between the two acids, as follows:

$$CH_3CO \cdot SH\ (g) + \tfrac{1}{2}O_2\ (g) \rightarrow CH_3 \cdot CO \cdot OH\ (g) + S\ (\text{rhombic}) \qquad (I)$$
$$\Delta Hr' = -61.3 \text{ kcal.}$$

Further, from the heats of formation of gaseous *n*-pentanol [4,5], —71.3 kcal. mole^{-1}, and 1-pentanethiol [1], —26.7 kcal. mole^{-1}:

$$C_5H_{11} \cdot SH\ (g) + \tfrac{1}{2}O_2\ (g) \rightarrow C_5H_{11} \cdot OH\ (g) + S\ (\text{rhombic}) \qquad (II)$$
$$\Delta Hr'' = -44.7 \text{ kcal.}$$

By analogy, from ethanol [2] and ethanethiol [2]

$$C_2H_5 \cdot SH\ (g) + \tfrac{1}{2}O_2\ (g) \rightarrow C_2H_5 \cdot OH\ (g) + S\ (\text{rhombic}) \qquad (III)$$
$$\Delta Hr''' = -46.4 \text{ kcal.}$$

In a normal case, the substitution of an oxygen for a sulfur atom decreases the enthalpy of formation by an average of 45.5 kcal. In the case of the acids the decrease is 61.3 kcal. Thus, we can write

$$R_{HSAc} = R_{HOAc} - 16 \text{ kcal.} \quad (1)$$

where R_{HSAc} and R_{HOAc} are the resonance energies of thiolacetic and acetic acid, respectively.

To estimate the magnitude of the resonance energy for acetic and thiolacetic acid we proceed as follows: The resonance energy of acetic acid is found using the equation:

$$CH_3CH_2 \cdot OH\ (g) + \tfrac{1}{2}O_2\ (g) \rightarrow CH_3COOH\ (g) + H_2\ (g) \quad (IV)$$
$$\Delta Hr = -48.1 \text{ kcal.}$$

For the analogous reaction

$$CH_3 \cdot CH_2 \cdot CH_3\ (g)^7 + \tfrac{1}{2}O_2\ (g) \rightarrow CH_3COCH_3\ (g)^2 + H_2\ (g) \quad (V)$$
$$\Delta Hr = -27.0 \text{ kcal.}$$

A comparison gives immediately

$$R_{HOAc} = 21 \text{ kcal.}$$

in agreement with the result of 22 kcal., obtained by the group equivalent method [6]. From equation 1 $R_{HSAc} = 5$ kcal. is deduced.

The resonance energy of thiolacetic acid may be calculated using another approach. If we look at the acid as an acetone molecule, substituting a sulfur atom for a CH_2 group, we can write:

$$CH_3 \cdot CO \cdot CH_2 \cdot H\ (g) + S\ (rh) \rightarrow CH_3 \cdot CO \cdot SH\ (g) + C\ (graphite) + H_2\ (g) \quad (VI)$$
$$\Delta Hr = +9.2 \text{ kcal.}$$

Comparing it with

$$C_4H_9 \cdot CH_2 \cdot C_4H_9\ (g)^7 + S\ (rh) \rightarrow C_4H_9 \cdot S \cdot C_4H_9\ (g)^1 + C\ (graphite) + H_2\ (g) \quad (VII)$$
$$\Delta Hr = +14.4 \text{ kcal.}$$

we get directly

$$R_{HSAc} = 5.2 \text{ kcal.}$$

The difference between the resulting values R_{HOAc} and R_{HSAc} is 15.9 kcal., in excellent agreement with the 16 kcal. found above. According to the conventional procedure one calculates from bond energy term values and heats of atomization of the elements [8]

$$R_{HOAc} = 19.2 \text{ kcal. and } R_{HSAc} = 3.8 \text{ kcal.}$$

The difference, 15.4 kcal., is also in good agreement with the result from the direct comparison between acetic and thiolacetic acid.

We conclude, that the resonance energy of thiolacetic acid is small compared with that of acetic acid, the value being only 4—5 kcal. mole^{-1}.

Thiophene. A cyclic, resonating molecule like thiophene may be thought of as possessing energy properties deviating from those of a normal molecule, mainly due to strain and resonance. Sketching the thiophene atom sequence

in the most stable configuration and with the ring opened up we get essentially a *trans*-1-mercapto-1,3-butadiene. This molecule is also stabilized by resonance, but it can be said — by definition — to be unstrained *.

By an adequate comparison between the structure — CH = CH — CH = CH — S — and selected structural elements the resonance energy in the mercaptobutadiene can be estimated. From experiments one should also be able to determine the energy difference between the butadienethiol and thiophene. The ring closure introduces an extra stabilization due to increased resonance and, at the same time, a strain labilization is created. It is not possible to separate these two effects by thermochemical experiments and we are therefore forced to incorporate the strain energy term into the resonance energy term. In doing this we must consider it desirable to put the strain energy solely into the cyclic, resonating moiety and select corresponding standard structural elements as free from strain as possible. The argument may be illustrated by the following example:

In estimating the resonance energy of *cyclo*pentadiene from heats of hydrogenation by the Kistiakowsky method [9], it has often been advocated that the best choice of a comparable molecule would be *cyclo*pentene. Twice the heat of hydrogenation of *cyclo*pentene should be identical to the ΔHh-value of *cyclo*pentadiene, were it not for the presence of resonance in the lastmentioned molecule. Introducing $\Delta Hh'$ and $\Delta Hh''$ for the heats of hydrogenation of *cyclo*pentene and *cyclo*pentadiene, respectively; S and S' for the strain energy in *cyclo*pentane and *cyclo*pentene, R'' for the net stabilization energy (formally the difference resonance energy minus strain energy) of the *cyclo*pentadiene moiety and the subscripts real and ideal to denote the character of the process, we can write

$$C_5H_8\ (g) + H_2\ (g) \rightarrow C_5H_{10}\ (g) \qquad \text{(VIII)}$$
$$\Delta Hh'_{exp} = \Delta Hh'_{ideal} + S' - S$$

and

$$C_5H_6\ (g) + 2H_2\ (g) \rightarrow C_5H_{10}\ (g) \qquad \text{(IX)}$$
$$\Delta Hh''_{exp} = \Delta Hh''_{ideal} - R'' - S$$

Remembering that $\Delta Hh''_{ideal} = 2\ \Delta Hh'_{ideal}$, we get

$$2\Delta Hh'_{exp} - \Delta Hh''_{exp} = R'' + 2S' - S$$

Only if $2S' = S$ can the Kistiakowsky method give information on the net stabilization (resonance) energy of *cyclo*pentadiene.

The procedure may be used for the determination of the resonance energy of thiophene in the following reaction:

$$C_4H_4S\ (g) + H_2\ (g) \rightarrow C_4H_6\ (g) + S\ (\text{rhombic}) \qquad \text{(X)}$$
$$\Delta Hr = -1.1\ \text{kcal.}$$

The heat of formation of butadiene $\Delta Hf\ (g) = +26.33$ kcal. mole^{-1} is obtained from Ref.[2]. This reaction is compared to the following reaction:

$$C_5H_{10}S\ (g) + H_2\ (g) \rightarrow C_5H_{12}\ (g) + S\ (\text{rhombic}) \qquad \text{(XI)}$$

* A discussion concerning the experimental approach to the problem of determining strain and resonance energies and the definition of these concepts will be published later.

ΔHf (g) = —15.14 kcal. mole^{-1} for thia*cyclo*hexane [1] and —35.00 kcal. mole^{-1} for pentane [7].

$$\Delta Hr = -19.9 \text{ kcal.}$$

It has been shown [1] that thia*cyclo*hexane is without strain, pentane is evidently also strainless. The resonance energy of 1,3-butadiene is 3.5 kcal. mole^{-1} [9].

The difference between the ΔHr-values equals the resonance energy of thiophene less that of butadiene, thus R = 22.3 kcal. mole^{-1}.

A different approach gives a comparison with the indirectly obtained value for the heat of hydrogenation of thiophene to thia*cyclo*pentane:

$$C_4H_4S\ (g) + 2H_2\ (g) \rightarrow C_4H_8S\ (g)$$

ΔHf (g) = +27.5 kcal. mole^{-1} for thiophene and —8.1 kcal. mole^{-1} for thia*cyclo*pentane *

$$\Delta Hh = -35.6 \text{ kcal.}$$

However, the hydrogenation product, the thia*cyclo*pentane molecule, is strained. It has been found that thia*cyclo*hexane is unstrained [1] and from the heat of formation values of the five- and six-membered rings, —8.1 and —15.14 kcal., respectively, and the CH_2-increment in the heat of formation of *n*-alkanes, —4.926 kcal.[12], the strain energy of the ring is found to be 2.1 kcal. Thus, the heat of hydrogenation of thiophene to a hypothetical strainless thia*cyclo*pentane should have been —35.6—2.1 = —37.7 kcal. mole^{-1}. This result should be compared with the heat of hydrogenation of a *trans*-alkene (since the *cis*-alkene is 'strained' in comparison with the *trans*-alkene and we want to get strain energy only in the thiophene moiety). From the heat of formation of *trans*-3-hexene [7] and hexane [7] ΔHh = —27.4 kcal. mole^{-1} is obtained. Combining this value and the ΔHh-value for thiophene to a strainless thia*cyclo*pentane one gets R = 17.1 kcal. mole^{-1}.

In the literature the resonance energy of thiophene is usually given as about 30 kcal. However, using Cottrell's thermochemical bond energy terms and the sulfur-carbon bond energy term from Ref.[1] one gets R = 22 kcal. According to the group equivalent method, using data given by Franklin [13] R becomes also 22 kcal. From Pauling's data in the same way R = 20 kcal. is obtained. The best estimate of the resonance energy of thiophene is probably therefore 20 kcal. mole^{-1}.

Thianthrene. The heat of formation of thianthrene has been determined to be: $\Delta Hf°$ (s) = +43.15 kcal. mole^{-1} **.

The heat of sublimation of thianthrene was estimated to be 20 kcal. mole^{-1} from a comparison between vapor pressure data of naphthalene, anthracene and phenanthrene [5], combined with melting and boiling temperatures of these

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* Ref.[10]. The author obtained —6.6 kcal [11]. The difference is partly due to an erroneous value of the heat of vaporization. The USBM-value is believed to be more reliable and it has therefore been used here.

** Average of the values obtained at this laboratory [14] and at US Bureau of Mines, Bartlesville [10].

compounds and thianthrene. Klages' equation gives 18.5 kcal.[15] Thus, ΔHf (g) = +63 kcal. mole^{-1}.

The resonance energy of thianthrene in excess of that of the two benzene nuclei is obtained from the following formal reaction.

$$C_{12}H_8S_2\ (g) + 2H_2\ (g) \rightarrow 2C_6H_6\ (g) + 2S\ (\text{rhombic}). \qquad (XIII)$$

ΔHf (g) = 19.8 kcal. mole^{-1} for benzene [7].
$\Delta Hh'$ = —23 kcal.

For comparison we chose reaction (XI) above.

$$C_5H_{10}S\ (g) + H_2\ (g) \rightarrow C_5H_{12}\ (g) + S\ (\text{rhombic}) \qquad (XI)$$

$$\Delta Hh'' = -19.9 \text{ kcal.}$$

Thus, $\Delta Hh'$—2 $\Delta Hh''$ becomes 17 kcal. which equals the resonance energy of thianthrene in excess of that of the two benzene nuclei. Thus, the total resonance energy of thianthrene is 89 kcal. (The resonance energy of benzene, 36 kcal., is the value obtained by use of the group equivalent method [6]). Using the thermochemical bond energy terms on reaction (XIII) one also gets R = 17 kcal. and 89 kcal., respectively.

Thiourea and thiosemicarbazide. In an early paper from this laboratory the heats of combustion for thiourea and urea [16] were given. Although the results were not very accurate they have been recalculated according to later requirements [11]. The value of the heat of combustion of crystalline thiourea to water, carbon dioxide and rhombic sulfur thus becomes ΔEc° = —209.4 kcal. mole^{-1} and the heat of formation is calculated to ΔHf (c) = —21.4 kcal. mole^{-1}. (*Selected Values* [2] gives —22.1 kcal.). The heat of sublimation has been roughly estimated to be 15 kcal. mole^{-1}. Pauling used ΔH_{vap} = 18 kcal. mole^{-1} for urea [17] and Wheland's estimate [9] was 16 kcal. mole^{-1}. Considering the differing hydrogen bonding in the two substances estimated to 6 kcal. mole^{-1} for urea and 1 kcal. mole^{-1} for thiourea and the *ca.* 2 kcal. higher ΔH_{vap} value for a C=S compound compared with the corresponding oxygen analogue, the difference between the heats of sublimation of urea and thiourea was estimated to be 3 kcal. mole^{-1}. The heat of formation of gaseous thiourea thus becomes ΔHf (g) = —6 kcal. Using thermochemical bond energy and heat of atomization values from Cottrell [8], we get

$$E_{C=S} + R_{\text{thiourea}} = 142 \text{ kcal.}$$

where R_{thiourea} is the resonance energy. Comparing the analogous equations for CS_2, COS and MeCNS [1] we find:

$$E_{C=S} + R_{MeNCS} = 118 \qquad (1)$$
$$E_{C=S} + 0.5\ R_{CS_2} = 121 \qquad (2)$$
$$E_{C=S} + R_{COS} = 134 \qquad (3)$$
$$E_{C=S} + R_{\text{thiourea}} = 142 \qquad (4)$$

The equations clearly show that the interaction energy increases in the series from MeNCS to thiourea. This is an obvious reason for treating the bond energy

term and the resonance energy term separately (*cf.* previous paper). Equations (1)—(3) were used to deduce the C=S bond energy value 115 kcal. mole^{-1}. Substituting this value for E_{C-S} in equation (4) we get

$$R_{\text{thiourea}} = 27 \text{ kcal.}$$

The heat of formation of thiosemicarbazid has been deduced from its heat of combustion, determined in this laboratory *.

$$\Delta Hf \text{ (c)} = +5.8 \text{ kcal. mole}^{-1}.$$

The heat of vaporization is very roughly estimated to 19 kcal. mole^{-1} (from boiling point differences between amines and hydrazines, Klages' equation for ΔH_{vap} at 25° C, and the value 16 kcal. for the heat of sublimation of thiourea). Thus ΔHf (g) = 25 kcal. mole^{-1} for gaseous thiosemicarbazide. By a calculation similar to that used for thiourea we obtain (E_{N-N} = 21 kcal.[8], R_{tsd} is the resonance energy of thiosemicarbazid):

$$E_{C-S} + R_{tsd} = 143$$

The value is almost identical with that obtained for thiourea. This is also to be expected, as the electrons on the 1-nitrogen atom do not participate in the resonance of the NH—CS—NH_2-system. The resonance energy of thiosemicarbazid thus becomes 28 kcal. mole^{-1}.

HEATS OF COMBUSTION

Materials. The thiophene was obtained in a vacuum sealed glass ampoule from US Bureau of Mines, Thermodynamics Laboratory, Bartlesville, USA, as "Samples of API — BM certified sulfur compounds purified at the Laramie Station of the Bureau of Mines and made available by American Petroleum Institute Research Project 48 A on the Production, Isolation and Purification of Sulfur Compounds and the Measurement of their Properties" **. The purity of the thiophene was given as 99.96 ± 0.02 mole-%.

The ampoule was sealed to a Pyrex-glass system, the break-off seal broken in vacuum and the thiophene distilled into the tube where the small glass ampoules for the combustions were placed.

The thiolacetic acid was synthesized according to a method recently described by Sjöberg [18] and distilled five times *in vacuo* in an atmosphere of nitrogen. The main fraction had a b.p. range of 0.01° C; the determination of condensation point differences had to be abandoned due to the catalytic decomposition of the substance in the presence of platinum [19].

Immediately after the last distillation, the ampoules were filled *in vacuo* and the combustions performed without delay ***.

Apparatus and method; Units of measurement and auxiliary quantities; Energy equivalent of the calorimeter; Heat of combustion of paraffin oil and Correction for the heat of formation of sulfuric acid. See Ref.[1].

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*** Data for the main fraction before the last distillation: B. p. 61.42 at 310 mm Hg, n_D^{20} 1.4648; d_4^{20} 1.0689.

RESULTS OF COMBUSTION EXPERIMENTS **

S_B = 5251.3 cal. per I.U., 5255.1 cal. (x).

Sample mg mass	Oil mg mass	ΔT_{corr} I.U.	q_{fuse} cal.	q_N cal.	q_{CO_2} cal.	$\Delta Ec°/M$ cal. g mass^{-1}
			Thiophene.			
	M = 84.138; D_4^{20} 1.065 S_F = 0.5 cal., q_{Wc} = 0.7 cal.					
362.97 x	214.26	1.00888	14.7	10.7	6.0	6315.4
320.19	242.21	1.00341	16.2	10.5	5.9	6315.9
345.83	220.22	0.99602	14.4	10.7	6.0	6315.0
452.72 x	144.58	0.99975	14.6	10.5	6.3	6315.9
403.21 x	173.13	0.98376	13.4	9.9	6.1	6315.8
					Average	6315.6
			Thiolacetic acid.			
	M = 76.118; D_4^{20} 1.0689 S_F = 0.7 cal., q_{Wc} = −0.3 cal.					
244.38	345.85	0.98612	15.1	10.0	5.0	3584.0
203.33	364.42	0.98181	16.0	10.4	5.0	3580.3
219.48	361.33	0.99203	14.3	10.0	5.1	3587.6
					Average	3584.0

For the reaction

$$C_4H_4S\ (liq) + 5O_2\ (g) \rightarrow 4CO_2\ (g) + 2H_2O\ (liq) + S\ (rhomb) \quad (XV)$$

$\Delta Ec° = -531.38 \pm 0.02$ kcal. mole^{-1} and
$\Delta Hc° = -531.96 \pm 0.02$ kcal. mole^{-1}.

At 25° C the heat of combustion of thiophene at unit fugacity is (heat capacities from Refs.[2,20]):

$\Delta Hc° = -531.97$ kcal. mole^{-1}

The heat of formation of liquid thiophene at 25° C is thus

$$4C\ (graphite) + 2H_2\ (g) + S\ (rhomb) \rightarrow C_4H_4S\ (liq) \quad (XVI)$$

$\Delta Hf°\ (liq) = 19.13 \pm 0.15$ * kcal. mole^{-1}.

The heat of vaporization of thiophene at 25° C is 8.29 kcal. mole^{-1} according to Waddington *et al.*[20].

Thus the heat of formation of gaseous thiophene at 25° C and unit fugacity from the elements in their thermodynamic standard states is $\Delta Hf°\ (g) = 27.4$ kcal. mole^{-1}.

For the reaction

$$2C\ (graphite) + 2H_2\ (g) + \tfrac{1}{2}O_2\ (g) + S\ (rhombic) \rightarrow CH_3COSH\ (liq) \quad (XVII)$$

$\Delta Hf°\ (liq) = -51.5 \pm 0.35$ ** kcal. mole^{-1} at 20° C.

From the boiling points measured at different pressures, the heat of vaporization is estimated to be 8.9 ± 0.3 kcal. mole^{-1}. It had to be assumed that gaseous thiolacetic acid is monomolecular. Spectroscopic investigations[21,22]

* Legend, see previous paper, Ref.[1].
** Twice the "overall" standard deviation.

have established, that the hydrogen bonding effect of the thiolcarboxyl group is very weak. One may conclude that the association in the gas phase is at least much less pronounced for thiolacetic acid than for acetic acid and so the error introduced cannot be of real significance for the calculation of the resonance energy.

The heat of formation of gaseous thiolacetic acid was thus computed to be

$$\Delta Hf = -42.6 \text{ kcal. mole}^{-1}.$$

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Short Communications

Studies on Keto Acids of Normal and Cancerous Tissues of Rabbit

P. LINKO and ARTTURI I. VIRTANEN

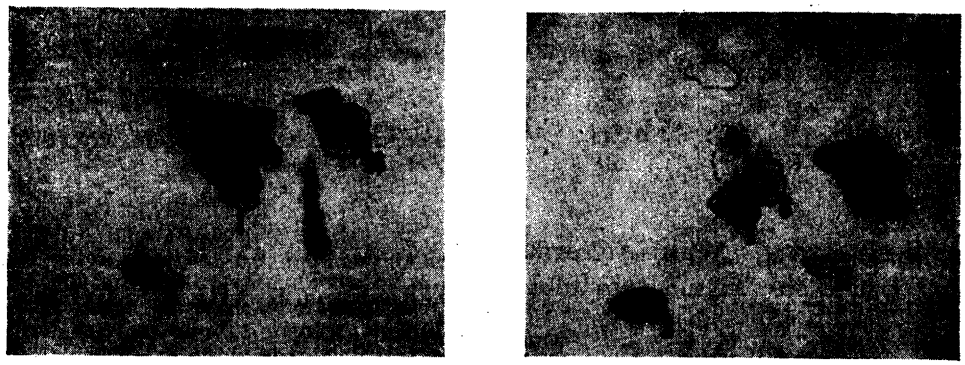
Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Virtanen and Alfthan [1-3] have previously determined the keto-acids in plants by reducing their 2,4-dinitrophenylhydrazones with tin in an alcoholic solution of hydrochloric acid to the corresponding amino acids, which were then determined paperchromatographically. The present authors have now applied the same method for examining animal tissues. Kulonen [4] determined the keto-acids of urine by a similar method using as reducing agent aluminium amalgam. He could show the presence of pyruvic, glyoxalic and α-ketoglutaric acids, the method, however, giving very unsatisfactory yields with dicarboxylic acids. The serine, which he could recognize on his chromatograms, he explained to be an artefact from aldol-condensation products of pyruvic acid [5]. Serine has also been found by the present authors among the reduction products of the keto acid 2,4-dinitrophenylhydrazones from both plant [1-3] and animal tissues, but under these reaction conditions no aldol condensation can be thought to occur. The pure 2,4-dinitrophenylhydrazones of pyruvic, aspartic and glutamic acids were also reduced but the only artefacts which were found were some alanine from oxaloacetic acid (about 3 %) and sometimes a very faint spot of γ-aminobutyric acid from α-ketoglutaric acid.

Adams' platinum oxide catalyst was also tried as reducing agent and, when the reduction was carried out at room temperature and 1 atm. pressure, somewhat better yields were obtained in the cases of the dicarboxylic acids than was found by tin reduction (about 50 % from α-ketoglutaric and 35 % from oxaloacetic acid). When starting from pure 2,4-dinitrophenylhydrazones (1 mg/ml in abs. ethanol) and slightly increasing the amount of the catalyst the reduction time was reduced from 2—3 hours to about 3 minutes and the solution did not darken at all when standing in air after reduction. The yield in these cases was as high as about 80—90 % from α-ketoglutaric and 50—60 % from oxaloacetic acid. From the former no γ-aminobutyric acid could be detected in these conditions and from the latter only about 2 % of alanine. The yields from other synthetical 2,4-dinitrophenylhydrazones examined were similar, irrespective of the reduction method used, varying from 15 to 50 %.

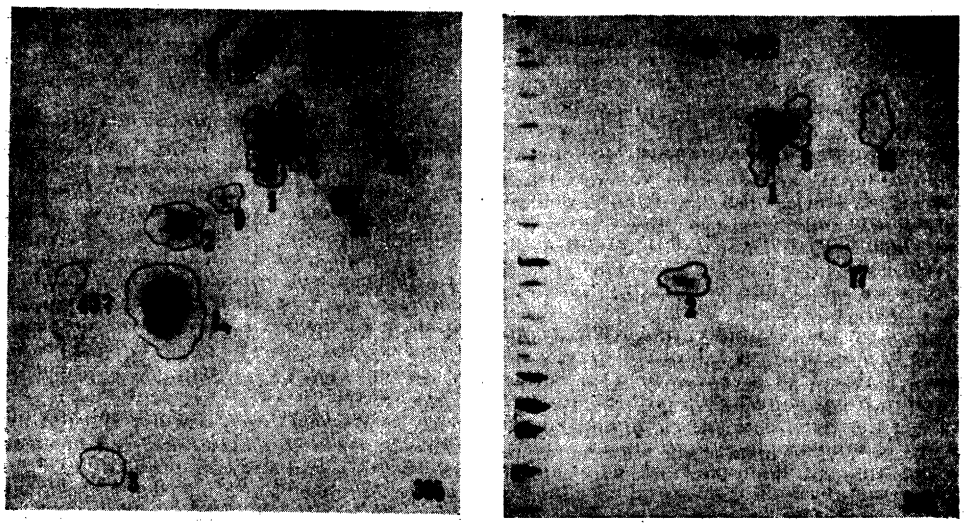
However, when attempting to apply the catalytical reduction to animal tissues, the catalyst was rapidly inactivated to a considerable extent. From muscle and kidney the yields were much lower than after reduction with tin, and from liver extracts no amino acids could be obtained by catalytical reduction. Therefore we found the reduction by tin as the best hitherto known method for this purpose.

The cancer was developed by treatment of the right ear of the rabbit with a 0.3 % solution of 9,10-dimethyl-1,2-benzanthracene in acetone every second day using 15 to 20 drops each time. The left ear was used as a control and quite healthy rabbits were examined too. Some determinations were also made on kidney and liver. In every case large amounts of pyruvic acid and somewhat smaller amounts of glyoxylic, hydroxypyruvic, oxaloacetic and α-ketoglutaric acids were found. In most cases no notable differences between normal and cancerous tissues could be found. In those cases where differences occurred they seemed accidental. No regular differences between normal and cancerous tissues could thus be observed on the basis of the material used. Le Page [6] has found somewhat higher amounts of pyruvic and α-ketoglutaric acid in cancerous tissues than in normal ones.



A B

Fig. 1. Two-dimensional chromatograms of amino acids formed by reduction of keto acid 2,4-dinitrophenylhydrazones from normal (A) and cancerous (B) ears of a rabbit. 1 = gly, 2 = ala, 8 = ser, 16 = asp, 17 = glu.



A B

Fig. 2. Two-dimensional chromatograms of amino acids formed by reduction of keto acid 2,4-dinitrophenylhydrazones from normal (A) and cancerous (B) ears of a rabbit. L = unknown amino acid, 9 = threo, 3 = val.

In one case, a remarkable amount of glyoxalic acid was found in the normal ear, but in the cancerous ear only normal a concentration of this acid, and instead a very high concentration of oxaloacetic acid (Fig. 1, A and B). In another case a high concentration of an unknown keto-acid was found in the normal ear, which could not be found in the cancerous ear (Fig. 2, A and B). The ninhydrin reaction of the corresponding amino acid was at first similar to that of tyrosine, the colour rapidly changing to normal violet. In a few other cases very faint spots of this substance were

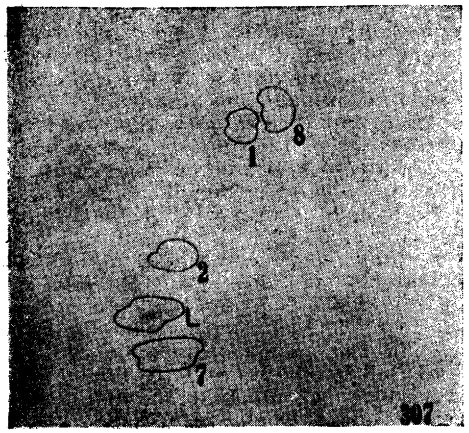


Fig. 3. Same as Fig. 2A, but tyrosine (spot 7) is added to the chromatogram.

found too. The amino acid is certainly not identical with tyrosine (Fig. 3).

In kidney about the same amounts of keto-acids were found as in muscle (calculated on fresh wt.), but in liver only 1/8 of this quantity. In all cases hydroxypyruvic acid was found, as mentioned earlier. Thus this acid is now for the first time shown to exist free in animal tissues. However Sprinson and Chargaff [7] noticed that hydroxypyruvic acid is formed in slices of rat kidney as an oxidative deamination product of serine.

In some cases small spots of threonine (from β-hydroxy α-ketobutyric acid) and tyrosine (from phenylpyruvic acid) were found, but the existence of the corresponding keto-acids in nature could, however, not be proved because of the poor material at our disposal.

Our best thanks are offered to Professor Kai Setälä and Phil.mag. Sinikka Lundbom for the material and preparation of the cancerous tissues, as well as for the microscopic preparations. We are also indebted to Miss. Pirkko Kovakoski for technical assistance during this work.

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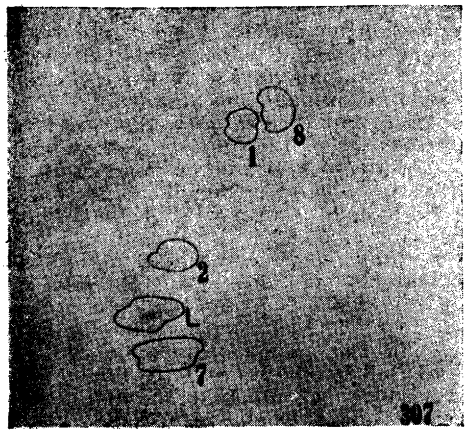
*iso*Thiocyanates XIII. Methyl *iso*-Thiocyanate, a New Naturally Occurring Mustard Oil, Present as Glucoside (Glucocapparin) in *Capparidaceae*

ANDERS KJÆR, ROLF GMELIN and IVAN LARSEN

Chemical Laboratory of the University of Copenhagen, Denmark

In a previous communication of this series [1] attention was given to a thiourea of extraordinarily low R_{Ph}-value (0.03) [2], derivable from the volatile *iso*thiocyanate fraction of certain seed samples. The mustard oil was tentatively regarded as methyl *iso*thiocyanate which had not previously been encountered in Nature. This suspicion has now been confirmed by the isolation of methylthiourea from the ammonia-treated distillate of extracts of seeds of *Cleome spinosa* Jacq. (*C. pungens* Willd.) after enzymatic hydrolysis.

The older literature [3] reports the use of various members of *Capparidaceae* as spices and remedies in folk medicine. These applications have been partly attributed to the contents of volatile constituents of unknown structure. In a previous paper [1] the species *Cleome arabica* L. and *Gynandropsis gynandra* (L.) Briq. were listed as sources of the presumed methyl *iso*thiocyanate, the latter being particularly rich in its contents. Methanolic extracts of various species of *Capparidaceae* have now been submitted to paperchromatographic investigation of their content of *iso*thiocyanate glucosides, essentially by the method of Schultz and Gmelin [4,5]. The results are schematically reproduced in Fig. 1. It should be noted that two glucosides with widely differing R_F-values appear



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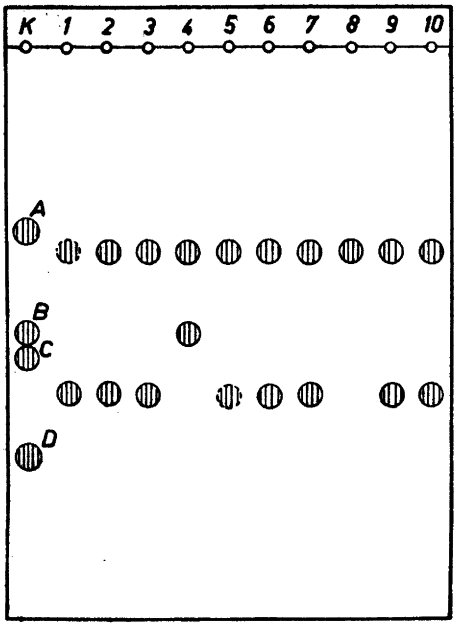

Fig. 1. Schematic, descending paper chromatogram of isothiocyanate glucosides *in various* Capparidaceae. *Solvent system:* n-*butanol:acetic acid:water (4:1:3). K represents a control mixture of: A) glucoiberin*[5] *B) sinigrin; C) sinalbin; D) glucotropaeolin. The following species are represented:*

1. Cleome arabica *L.*
2. C. arborea *Bss.*
3. C. gigantea *L.*
4. C. monophylla *L.*
5. C. speciosissima *Deppe.*
6. C. spinosa *Jacq.*
7. C. viscosa *L.*
8. Gynandropsis gynandra *(L.) Briq.*
9. Cleome graveolens *Rafin.*
10. C. trachysperma *(Torr. & Gray) Pax & K. Hoffm.*

to represent the general pattern of this family, occasionally accompanied by an additional glucoside as in, *e. g.*, *C. monophylla* L. The relative amounts of the two glucosides in various species show some variation but generally they appear to be present in about equal amounts.

When a larger sample of seeds of *C. spinosa* Jacq. (125 g) became available it was treated with myrosinase whereafter a sharp and typical smell of mustard oil soon developed. The water-soluble oil was removed by steam distillation and collected in ammonia. After standing, the solution was concentrated to dryness *in vacuo* leaving a semi-crystalline residue (290 mg), which was transformed into beautiful, dense crystals upon three recrystallizations from acetone. M. p. 121° (uncorr.), alone or in admixture with an authentic specimen of N-methylthiourea. (Found: C 26.54; H 6.43; N 30.84. Calc. for $C_2H_6N_2S$: C 26.65; H 6.71; N 31.09).

For the glucoside, yielding methyl *iso*thiocyanate on enzymatic cleavage, we should like to suggest the name *glucocapparin*. The fact that *C. monophylla* L., lacking the second main glucoside, on cleavage, distillation and treatment with ammonia also afforded methylthiourea proved the lower glucoside-spot of Fig. 1 to be attributable to glucocapparin, a constant constituent throughout the botanical family. The chemical nature of the second characteristic glucoside of higher R_F-value as well as of the second component in *C. monophylla* is at present being further investigated. It is obvious, however, that both substances represent glucosides yielding non-volatile mustard oils on enzymatic hydrolysis.

No definite proof of the presence of glucocapparin in *Cruciferae* has yet been found. The examples previously listed [1] have needed some revision which will be presented in forthcoming communications.

The authors wish to thank the Botanical Garden of the University of Copenhagen for numerous seed samples. Seeds of *C. spinosa* were commercially obtained from E. Benary, Hann.-Münden. The work is part of investigations supported by *Statens Almindelige Videnskabsfond (The Danish State Research Foundation)* and *Carlsbergfondet (The Carlsberg Foundation)*.

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Enzymatic Cleavage of S-Adenosylhomocysteine and the Transfer of Labile Methyl Groups *

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Evidence has recently been presented that S-(5'-desoxyadenosine-5')-homocysteine, referred to hereafter as S-adenosylhomocysteine, is the primary product resulting from methylations involving S-adenosylmethionine ("active methionine")[1]. S-adenosylhomocysteine has been synthesized by Baddiley and Jamieson [2]. Some experiments with synthetic S-adenosylhomocysteine, kindly supplied by Dr. Baddiley, The Lister Institute of Preventive Medicine, London, have been carried out in our laboratory in order to elucidate the metabolic fate of this compound.

The possibility existed that S-adenosylhomocysteine, and not homocysteine, might be the correct substrate for the methionine-generating enzyme, betaine-homocysteine transmethylase. This enzyme has recently been purified [3] and a comparison could thus be made of the amount of methionine formed from a given amount of S-adenosylhomocysteine (1.1 mg) with that produced by the same preparation of purified enzyme from an equivalent amount of homocysteine (0.35 mg). Methionine was determined by the colorimetric method of McCarthy and Sullivan [4]. It was found, using purified betaine-homocysteine transmethylase, that the yield of methionine from S-adenosylhomocysteine under aerobic or anaerobic conditions was negligible compared to that obtained from homocysteine. On the other hand with whole rat liver homogenate S-adenosylhomocysteine was observed to possess up to 40 % of the activity of homocysteine.

To study the reaction products formed enzymatically during the incubation of S-adenosylhomocysteine with whole rat liver homogenate, 0.55 mg of substrate and 0.1 ml of a 20 % rat liver homogenate (in 0.04 *M* phosphate buffer of pH 7.3) were incubated for 6 hours at 37° C in a total volume of 0.5 ml. Separation of the reaction products by one-dimensional paper chromatography revealed a ninhydrin-positive spot with the same R_F value as homocysteine in two different solvent systems [5,6]. When S-adenosylhomocysteine and rat liver homogenate were incubated together with betaine, it was observed that the spot with the same R_F as homocysteine decreased in intensity, and a spot with an R_F value identical with that of methionine in the two solvent systems appeared. A spot with the same R_F value as α-aminobutyric acid in the two solvent systems referred to above [5,6] as well as in a third system (2-methoxyethanol : propionic acid : water, 60:20:20 v/v) was also detected. The intensity of this spot was slightly reduced when betaine was added to the incubation mixture. Also, a ninhydrin-positive compound with the same R_F value as α-aminobutyric acid appeared on the chromatograms after incubation of either homocysteine or homoserine with rat liver homogenate, and the intensity of this spot was increased by addition of glutamate to the incubation mixture. No homoserine could be found in the incubation mixtures containing S-adenosylhomocysteine and rat liver homogenate. These observations indicate that, in the experiments with S-adenosylhomocysteine, α-aminobutyric acid was formed mainly from the homocysteine resulting from the enzymatic cleavage of S-adenosylhomocysteine. The identification of α-aminobutyric acid as one of the reaction products of the enzymatic degradation of S-adenosylhomocysteine offers an explanation for the finding by Dent [7] that human beings fed a large dose of methionine excrete appreciable amounts of α-aminobutyric acid in the urine.

In separate experiments using the purified apoenzyme, it was found that S-adenosylhomocysteine could not serve as a cofactor [3] for betaine-homocysteine transmethylase.

Considering the results of other investigators (for references, see [8,9]) together with our own [3], an attempt has been made to summarize the present knowledge about transmethylation reactions in animals (Fig. 1). It is likely that the formation of anserine [10] and of adrenalin [11,12] could be

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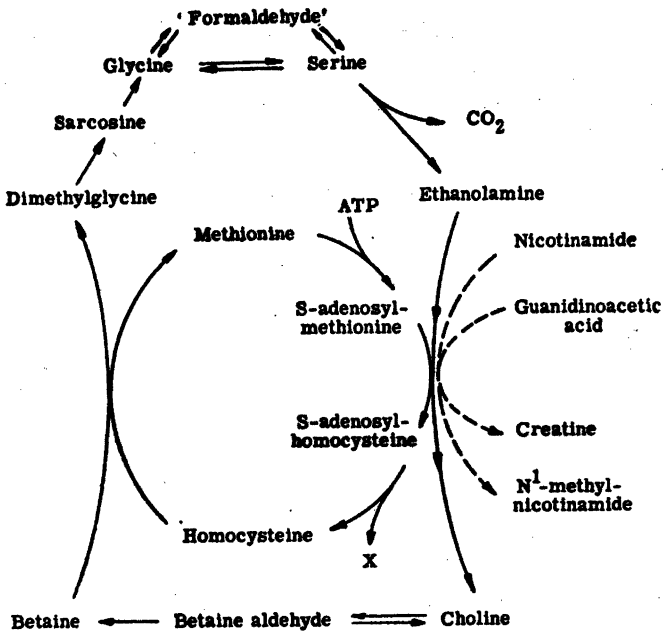

included in Fig. 1 in a manner similar to that used for creatine and N′-methylnicotinamide. It is also possible that dimethylthetin and dimethylpropiothetin should be included in this diagram as donors of methyl groups to homocysteine [13] in the same way as shown for betaine.

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The Structure and Bond Type of Co^{II} Chelates of 7-Iodo-8-Quinolinol-5-Sulphonic Acid

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During recent investigations on metal chelates of 8-quinolinol-5-sulphonic acid, Näsänen and Uusitalo [1] have observed that aqueous solutions of $Co^{II}(C_9H_4O_4NS)$ are subject to some change with time. A similar effect has also been observed in the Co^{II} chelate of 7-iodo-8-quinolinol-5-sulphonic acid (Ekman [2]). The yellow colour of the solution becomes intensified and the shape of the ultraviolet absorption spectrum changes, while its maximum shifts towards longer wavelengths. The phenomenon indicates the formation of a new complex compound, which has been assumed to have the composition $X_2[Co^{II}(C_9H_4O_4NIS)_2]$. The structures of these two Co^{II} chelates are represented in Fig. 1.

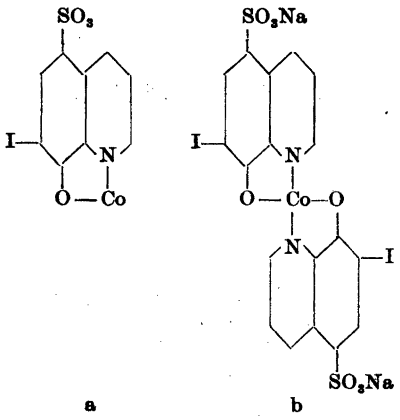

Fig. 1. The structure of $Co^{II}(C_9H_4O_4NIS)$ (a) and $Na_2[Co^{II}(C_9H_4O_4NIS)_2]$ (b).

The magnetic moment of $Co^{II}(C_9H_4O_4NIS) \cdot 2\ H_2O$ has been found to be 4.77 BM and that of $Na_2[Co^{II}(C_9H_4O_4NIS)_2] \cdot 4\ H_2O$ to be 1.70 BM. (The values of the magnetic moments are preliminary).

The magnetic criterion shows that the bond type in $Co^{II}(C_9H_4O_4NIS)$ is, as expected, essentially ionic while the bond type in $Na_2[Co^{II}(C_9H_4O_4NIS)_2]$ is essentially covalent, corresponding to one unpaired electron. There are two possible configurations of Co^{II} complexes which have one unpaired electron, namely the square planar (coordination number 4) and the octahedral (coordination number 6) structures. The especially low value of the magnetic moment could be interpreted as indicating the octahedral structure [3]. Since there are only two $C_9H_4O_4NIS$ groups to one Co atom, the octahedral structure should have the composition $Na_2[Co^{II}(H_2O)_2(C_9H_4O_4NIS)_2] \cdot 2\ H_2O$. By heating the substance to 130° C all the crystal water can, however, be removed without any marked change in the magnetic moment. It is therefore assumed that the coordination number is four and the structure square planar, involving $3d4s4p^2$ orbitals in the bond formation.

The ultraviolet absorption spectra of the compounds are given in Fig. 2. The broken line in Fig. 2a represents the molar extinction of $Co^{II}(C_9H_4O_4NIS)$ in 1 *M* HCl and the dotted line that of the chelating agent $C_9H_6O_4NIS$. The curves show that the freshly prepared chelate has decomposed to the chelating agent. In Fig. 2b the two curves, representing the molar extinction of $Na_2[Co^{II}(C_9H_4O_4NIS)_2]$ in distilled water and in 1 *M* HCl are similar, showing that the covalent compound is stable even in relatively strong mineral acids.

Preparation of compounds. $Co^{II}(C_9H_4O_4NIS)$ can be prepared by adding some soluble Co^{II} salt to a neutralised solution of 7-iodo-8-quinolinol-5-sulphonic acid. The precipitate contains transparent brown-red crystals of the composition $Co^{II}(C_9H_4O_4NIS) \cdot 3\ H_2O$. On heating the compound to about 40° C the third molecule of the crystal water is irreversibly removed, giving yellowish brown crystals. At about 130° C the rest of the crystal water is reversibly removed while the substance turns greenblack. The experiments were made with the substance $Co^{II}(C_9H_4O_4NIS) \cdot 2\ H_2O$.

$Na_2[Co^{II}(C_9H_4O_4NIS)_2] \cdot 4\ H_2O$ can be prepared by dissolving equivalent amounts of solid $Na_2C_9H_4O_4NIS \cdot 2\ H_2O$ and $Co^{II}(C_9H_4O_4NIS) \cdot 2\ H_2O$ in water. The reaction between the compounds is slow. By raising the temperature to about 40—50° C (higher temperatures were avoided to prevent decomposition of the compounds [4]) the reaction can be carried

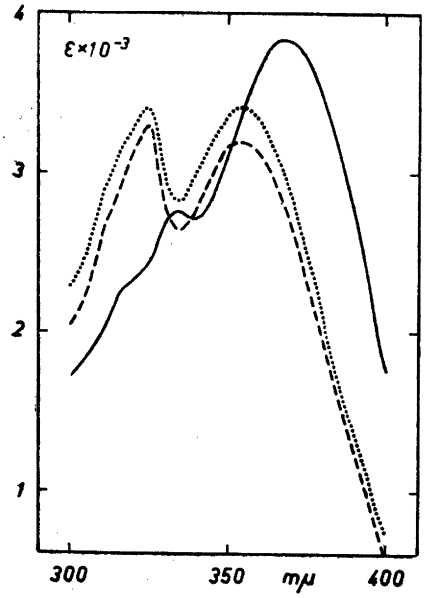

Fig. 2a. The molar extinction of $Co^{II}(C_9H_6O_4NIS)$ in distilled water ————, in 1 M HCl — — — — and the molar extinction of $C_9H_6O_4NIS$ in 1 M HCl .....

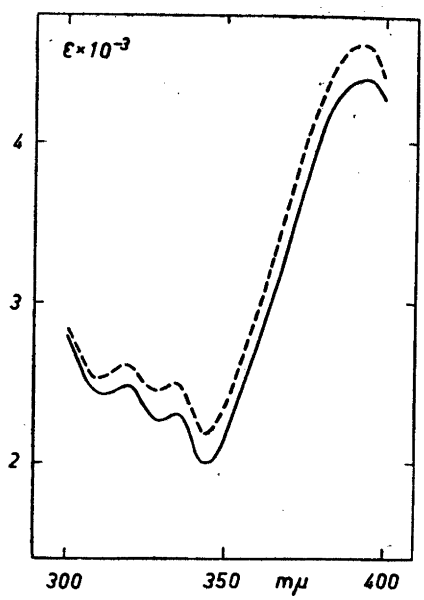

Fig. 2b. The molar extinction of $Na_2[Co^{II}(C_9H_6O_4NIS)_2]$ in distilled water — — — and in 1 M HCl ————

out more rapidly but not quantitatively. After repeated evaporations an almost black precipitate can be isolated. According to magnetic and spectrophotometric measurements the composition and structure of the precipitate is given by the formula $Na_2[Co^{II}(C_9H_6O_4NIS)_2]$. The compound is very soluble in water and soluble in alcohol. The solubility is not incompatible with the covalent bond because of the SO_3 groups [5]. The corresponding acid $H_2[Co^{II}(C_9H_6O_4NIS)_2]$ can be prepared by exchange reaction from the sodium salt. Potentiometric titration of the acid dissolved in water has shown it to be a strong acid.

Similar compounds with 8-quinolinol-5-sulphonic acid and its other derivatives as chelating agent are expected.

Further investigations are in progress.

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Sialic Acid in Human Serum Protein and in Meconium

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The presence of sialic acids or closely related substances in many glycoproteins and in gangliosides has been indicated by more or less specific colour reactions. In some materials the presence of these substances has been definitively proved by their isolation in pure form and identi-

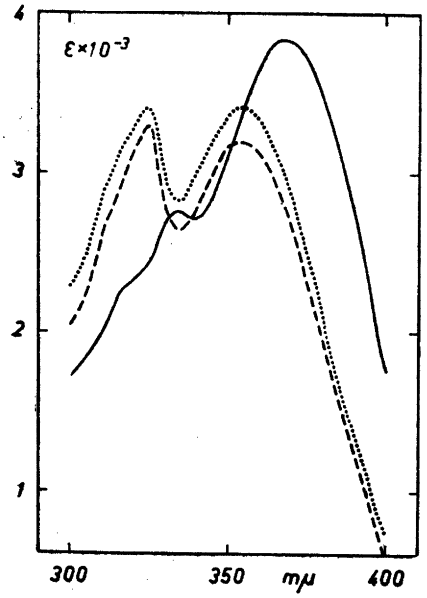
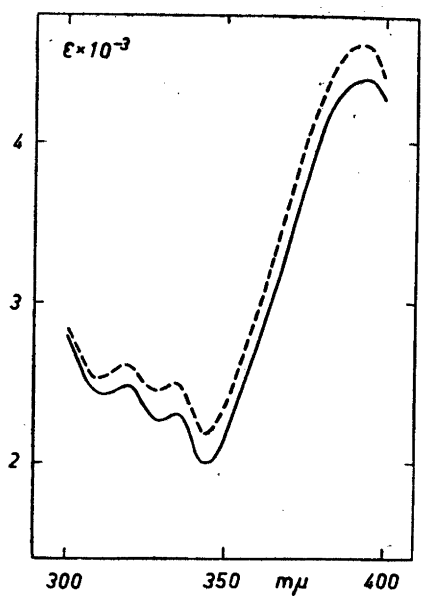


Fig. 2a. The molar extinction of $Co^{II}(C_9H_6O_4NIS)$ in distilled water ————, in 1 M HCl — — — — and the molar extinction of $C_9H_6O_4NIS$ in 1 M HCl .....



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fication by chemical and physical methods. In this paper the isolation of a sialic acid from human serum protein, an acid serum glycoprotein (orosomucoid [1]), and meconium will be reported.

In 1952 Werner and Odin [2], using *i. a.* the colour reactions with Bial's orcinol and Ehrlich's *p*-dimethylaminobenzaldehyde reagents, adapted for quantitative determination, showed that sialic acid most probably occurred in considerable amounts in certain serum glycoproteins, as human a_2-globulin, orosomucoid, and ox seromucoid. It was further observed, that the colour reactions with the diphenylamine and tryptophane-perchloric acid reagents, used by some authors in studies on normal and pathological sera (for references see Winzler [1]), as applied to blood serum probably were almost entirely due to the presence of sialic acid. In 1954 Böhm *et al.*[3], also using a quantitative adaption of the 'Bial' method, suggested the presence of neuraminic acid (Klenk) in human serum protein. Somewhat later Böhm and Baumeister [6] reported the isolation of methoxy-neuraminic acid in crystalline form from the same material.

Meconium has been reported to contain considerable amounts of glycoproteins with the chemical and serological properties of the blood group substances [5]. Using colour reactions Werner [4] has shown, that in addition to the blood group carbohydrates, sialic acid is present in varying amounts in all the mucous secretions investigated by him.

Experimental. Analytical methods. Nitrogen was determined by the Kjeldahl micromethod. For the hexosamine determinations Blix's modification [7] of the Morgan-Elson method was used. Hexose was determined by Vasseur's modification [8] of the Tillmanns-Philippi orcinol reaction. The values were corrected for the fucose content, determined by the method of Dische [9]. Sialic acid was assayed by the colour reactions with Bial's and Ehrlich's reagents [2]. Paper chromatographic analysis was performed with butanol-acetic acid and ethyl acetate-pyridine as solvents. The papers were sprayed with the orcinol-trichloroacetic acid reagent of Klevstrand and Nordal [10]. It gives violet spots with sialic acids. The X-ray powder diagrams were taken with a flat film camera, with a distance of 10.0 cm between the specimen and the film.

Materials. 2 l of pooled *human ACD-plasma* was dialysed for 96 hrs at 4° C, first against several changes of 0.01 *N* hydrochloric acid and then against distilled water. The proteins were precipitated by addition of 5 volumes of ethanol and dried with ethanol and ether. The dry powder contained 14.2 % nitrogen, 1.4 % hexosamine, 1.3 % hexose, <0.1 % fucose, 1.1 % sialic acid.

Orosomucoid was prepared as described by Weimer *et al.*[11] 2 g of the substance was obtained from 12 l of human ACD-plasma. The results of analysis corresponded well with those of earlier reported preparations [1,2]. By the chromatographic method of Gardell [12], however, glucosamine only, but no galactosamine, could be shown to be present. Analysis: 10.7% nitrogen, 12.2 % glucosamine, 13.5 % hexose, 1.4 % fucose, 10.6 % sialic acid.

Pooled human *meconium* was suspended in 0.01 *N* hydrochloric acid. After 48 hrs at 4° C under occasional stirring, the suspension was centrifuged. (Considerable amounts of carbohydrate were evidently still n suspension or dissolved in the supernatant.) The undissolved residue was resuspended in distilled water and dialysed. 5 volumes of ethanol were then added and the precipitate was dried with ethanol and ether. The greenish powder had the following composition: 10.1 % nitrogen, 11.8 % hexosamine, 7.9 % hexose, 4.9 % fucose, 5.0 % sialic acid.

For the isolation of sialic acid from these materials the method of Blix [13] was followed in principle. For the hydrolysis of the serum proteins weak sulphuric acid was used; simple heating with water apparently did not give sufficiently acid conditions for the splitting off of sialic acid. Sulphuric acid has earlier been used for the isolation of the lactaminic [14] and gynaminic [15] acids.

The serum protein preparation (90 g) and the orosomucoid (2 g) were heated for one hour on a boiling water bath with weak sulphuric acid (pH about 2). To the filtered solutions barium hydroxide was added in a little excess (pH about 8). After filtering the solutions were passed through cation exchange columns (Amberlite IRC-50, H) and then freeze-dried. The meconium dry powder (10 g) was heated with distilled water only, and the solution filtered and freeze-dried.

The dried materials were extracted with methanol and the extracts evaporated *in vacuo*. The residues were dissolved in methanol after addition of a little water. Ether was added in small portions for several days, the amorphous precipitates, which formed, being filtered off. When no more of these appeared, petroleum ether was added, and in a few days crystalline deposits were obtained. These

were recrystallized from a water-methanol mixture.

The crystalline materials from all three sources showed X-ray diffraction patterns identical with that of the sialic acid isolated by Blix *et al.*[16] from sheep submaxillary mucin and by Odin [17] from human ovarian cyst gels. The colour intensities with the 'Bial' and 'Ehrlich' reagents and the paper chromatographic R_F-values were also the same for all the substances.

Identical sialic acids have thus been isolated from three human sources, *viz.* pseudomyxomatous gels, serum proteins, and meconium, and from the submaxillary mucin of sheep. This substance differs from the sialic acids of ox, swine, and horse origin, which also differ between themselves [16].

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Separation of Saturated Straight Chain Fatty Acids. Qualitative Paper Chromatography

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Several different paper-chromatographic methods for the separation of saturated straight chain fatty acids or their alkali salts have been described in the literature. The methods published may be divided into two groups, the first of which involves the volatile acids (from formic to caproic acid) (Brown [1], Hiscox [2], Kennedy [3], Long [4], Isherwood [5]) and the second the long chain acids (from caprylic acid upwards) (Kaufmann [6], Inoyue [7], Spiteri [8], Baker [9], Wegman[10], Holasek[11], Kaufmann[12], Kobrle[13]). In the following, procedures now found suitable for the separation of fatty acids from formic to cerotic acid are described. Because of the volatility of the short chain acids and the poor solubility of the long chain acids not all the acids in question can be separated by one method, but they have to be analysed in separate groups. Suitable groups are: formic — caproic acid, caproic — capric acid, and capric — cerotic acid.

A very suitable method for the analysis of the first group, from formic to caproic acid, is that of Hiscox [2]. According to his method the acids are separated as their ammonium salts, using *n*-butanol saturated with water as solvent. The hydrolysis of the salts during the elution is prevented with ethylamine vapour. The elution is completed in a few hours when the circular chromatography method described by Rutter [14] is employed. A suitable chamber for this purpose was made by grinding two shallow glass bowls against each other and placing the paper between them. The inner diameter of the chamber was 33 cm and the inner height 4 cm. The acids are suitably detected by dyeing with the indicator mixture methyl red-bromothymol blue as described by Duncan [15]. Formic and acetic acid cannot be separated from each other by the method of Hiscox, but according to Lindqvist [16] they may be distinguished by using silver nitrate, which is reduced by formic acid, in connection with the identification. By these methods 0.05 mg of acid can easily be detected. The following approximate R_F-values were obtained:

were recrystallized from a water-methanol mixture.

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formic 0.37, acetic 0.38, propionic 0.52, butyric 0.65, valeric 0.75, caproic (hexoic) 0.86 and önanthylic (heptoic) acid 0.88.

The acids of the second group (from caproic to capric acid) may conveniently be separated by the method of Holasek [11], who used as solvent carbon tetrachloride-methanol-ammonia (81:18:1 v/v). Thus the acids migrate as ammonium salts. If a paper of 90 g/m² weight is used, not more than 0.05 mg of each acid can be separated. The acids are detected according to the method of Kaufmann [6] with a solution of Rhodamin-B; 0.01 mg of acid is still detectable. The best separation is achieved if the elution is continued until the undecoic acid has reached the edge of the paper. By the usual descending method the following R_F and R_U-values (R_U = undecoic acid as reference substance) were obtained: caproic (hexoic) 0.28 (R_F) and 0.57 (R_U), önanthylic (heptoic) 0.34 and 0.69, caprylic (octoic) 0.39 and 0.79, pelargonic (nonoic) 0.43 and 0.87, capric (decoic) 0.46 and 0.94, and undecoic acid 0.49.

Kaufmann [12] describes a reversed phase method for the separation of the even-numbered acids from capric to stearic acid. The paper is first saturated with hydrocarbon (bp. 190—220° C) and the elution is then carried out with acetic acid-water (9:1 v/v) using the descending method. This procedure was found in the present study to be easier to carry out when circular chromatograms are used. In these the paper need not be so evenly saturated as in the descending method and, of course, the process is shortened. With acetic acid-water all the even-numbered acids from capric to cerotic acid separated. Melissic (triacontoic) acid was not sufficiently soluble in these conditions and did not migrate on the chromatogram. Of the odd-numbered acids nonoic and undecoic acid were investigated and found to separate easily from each other and also from the even-numbered acids. Hence it is to be expected that all the other odd-numbered acids may likewise be separable from the even-numbered acids. On account of the small R_F-values the chromatograms must be eluted considerably longer than is needed for the solvent front to reach the edge of the paper. This is quite possible with the circular method now employed, because the solvent continuously evaporates from the edge of the paper and the elution may be prolonged indefinitely.

Since the third group includes a great number of acids, they could not be analysed on one chromatogram, because the size of the paper was limited by the size of the chamber used. When both even-numbered and odd-numbered acids were present, this group was further divided into three groups: capric—palmitic, palmitic—behenic, and behenic—cerotic acid. When only even-numbered acids were present, the division was made into two groups: capric—stearic, and stearic—cerotic acid.

After the hydrocarbon was evaporated from the paper, the acids were detected by converting them into their copper salts and then locating the copper as the ferrous cyanide complex (Kaufmann [12]); 0.1 mg of acid could thus easily be detected. In the conditions used, the R_F-values and R_C-values (capric acid as reference substance) of the acids were as follows: pelargonic (nonoic) 0.65 (R_F), capric (decoic) 0.51, undecoic 0.39 (R_F) and 0.77 (R_C), lauric (dodecoic) 0.32 and 0.63, myristic (tetradecoic) 0.24 and 0.48, palmitic (hexadecoic) 0.18 and 0.35, stearic (octadecoic) 0.13 and 0.26, arachidic (eicosoic) 0.10 and 0.19, behenic (docosoic) 0.07 and 0.14, lignoceric (tetracosoic) 0.06 and 0.11, and cerotic (hexacosoic) acid 0.05 and 0.09.

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A General Method for the Synthesis of Optically Active β-Hydroxy Acids

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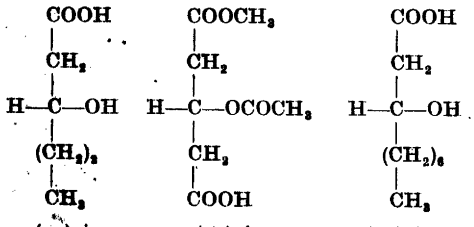
Racemic methyl hydrogen β-acetoxyglutarate, a viscous, slightly hygroscopic liquid with b.p. 150°/0.5 torr (slight decomposition), n_D^{25} 1.4470 and d_4^{25} 1.234, was made from the known anhydride of β-acetoxyglutaric acid [1,2] and methanol. The optically pure antipodes of this half-ester were obtained in about 30 % yield by fractional crystallization of the cinchonidine and strychnine salts from ethyl acetate and chloroform-ether, respectively. They are liquids with $[\alpha]_D^{25} \pm 6.1°$ (chloroform; *c* 20; *l* 1).

Electrolysis of the dextrorotatory antipode with propionic acid gave, *via* the acetylated methyl ester, β-hydroxyhexanoic acid (I) with m. p. 42° and $[\alpha]_D^{22} -28°$ (chloroform; *c* 2; *l* 1). As this acid is known to have the D configuration [3], formula II represents the Fischer projection of the dextrorotatory half-ester. It may be named (+)-methyl 3D-acetoxy-4-carboxybutanoate. *Cf.*[4,5].



(—) in chloroform (+) in chloroform (—) in chloroform (+) in in ethanol

I II III

Electrolysis of the levorotatory half-ester with *n*-heptanoic acid gave similarly 3 L-hydroxydecanoic acid with m. p. 48.4°; $[\alpha]_D^{22}$ +20° (chloroform; *c* 2.5; *l* 1); −3° (ethanol; *c* 2.5; *l* 1). The antipode of this acid has been isolated from natural sources [6-8] and it follows that this has the D-configuration (III). *Cf.*[3] A specimen kindly supplied by professor S. Bergström gave in equimolecular mixture with the synthetic acid the racemic 3-hydroxydecanoic acid with m. p. 56.6°. Skogh [9] reports m. p. 56.4–56.6°.

Apparently optically pure β-hydroxynonanoic with $[\alpha]_D$ +2° 26′ (ethanol) has been obtained by degradation of ricinoleic acid [10]. From the foregoing it seems highly probable that it has the D-configuration. Ricinoleic acid should thus be 12D-hydroxy-*cis*-9-octadecenoic acid.

It is obvious that the optically active half-ester (II) and its antipode can be used as starting material for the synthesis of a great many optically active hydroxy-compounds of the type $RCH_2CHOHCH_2R'$.

A full account of this work will be published later. Grants from *Norges Almenvitenskapelige Forskningsråd* and from *Statens Medicinska Forskningsråd* are gratefully acknowledged.

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On the Polarography of Formaldehyde *

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In the paper a mathematical treatment is given for the polarographic behaviour of formaldehyde in neutral and alkaline solutions. This treatment is co-ordinated with earlier, simplified solutions, which are valid at high rates of methylene glycol dehydration. The derivations are extended to give a method for determination of the equilibrium and dehydration rate constants when the limiting current is controlled both by catalysis and by diffusion. The effect of the hydroxyl ions produced at the electrode surface has been considered for the case of unbuffered or slightly buffered solutions. The theoretical conclusions are verified experimentally. The spectrophotometric equilibrium constant of methylene glycol—formaldehyde is checked experimentally and found to be in agreement with earlier published results.

Despite the fact that the polarographic determination of formaldehyde is a well-established analytical process, only simplified mathematical treatments are given for the formaldehyde polarographic current. The purpose of the following paper is to give a more rigorous treatment, in order to make it possible to find equilibrium and dehydration rate constants for the system formaldehyde—methylene glycol from experimental data. The rate of dehydration is of a considerable interest in discussions on the mechanism of several formaldehyde reactions, *e. g.* in the methylol urea synthesis.

WATER SOLUTIONS OF FORMALDEHYDE

According to *e. g.* Auerbach and Barschall [1], in concentrated water solutions formaldehyde is present as trioxymethylene glycol, $HO \cdot (CH_2O)_3 \cdot OH$. When diluted, this compound depolymerises to methylene glycol, $HO \cdot CH_2 \cdot OH$. The rate of the depolymerisation,

$$HO \cdot (CH_2O)_3 \cdot OH + 2\ H_2O = 3\ HO \cdot CH_2 \cdot OH \qquad (A)$$

has been studied by Wadano *et al.*[2], and it has been shown that it is dependent on pH, having a minimum at pH $\sim$ 3.5.

In the electrolytic dissociation of methylene glycol the following reactions probably occur:

$$HO \cdot CH_2 \cdot OH = H_2C^+ \cdot OH + OH^- \qquad (B)$$

$$HO \cdot CH_2 \cdot OH = H_2C^+ \cdot O^- + H^+ + OH^- \qquad (C)$$

$$HO \cdot CH_2 \cdot OH = HO \cdot CH_2 \cdot O^- + H^+ \qquad (D)$$

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Wadano [2] states that reaction (B) is of interest at pH $<$ 2.6, (C) at 2.6 $<$ pH $<$ 4.6 and (D) at pH $>$ 4.6. The dissociation constant of reaction (D) has been determined by *e. g.* Euler and Lövgren [3] and Lévy [4]. A large temperature effect has been observed, at 0° C pK = 14.0, at 20° C pK = 13.4 and at 50° C pK = 12.5.

A spectrophotometric study of the equilibrium between methylene glycol and formaldehyde

$$HO \cdot CH_2 \cdot OH = HCHO + H_2O \qquad (E)$$

has been made by Schou [5] in a 13 M solution and by Bieber [6] in a 0.86 M solution. Schou estimates the equilibrium constant to be $<$ 1/1250, and by means of extrapolation from measurements in a temperature range close to 60° C Bieber gets a probable constant value at 20° C of the order 10^{-4}.

THE POLAROGRAPHIC BEHAVIOUR OF FORMALDEHYDE

The polarographic reduction of formaldehyde at low temperatures (25° C) gives a limiting current which is less than that deduced from the concentration polarisation, *i. e.* from the Ilkovič [7] equation:

$$\bar{i}_d = 607\, n\, C \sqrt{D}\, m^{2/3}\, t_1^{1/6} \qquad (1)$$

where $\bar{i}_d$ = diffusion current in μA, n = number of Faradays of electricity required per mole of electrode reaction, C = concentration of the solution of the electroreducible substance in millimoles/l, D = diffusion constant of the same substance in cm^2/sec, m = weight of mercury flowing from the capillary in mg/sec and t_1 = drop time in seconds.

From comparisons between Mn^{2+} and formaldehyde at a high temperature (80° C), where the formaldehyde reduction also follows eq. (1), Jahoda [8] found the following electrode reaction:

$$HCHO + 2\ H_2O + 2\ e = CH_3OH + 2\ OH^- \qquad (F)$$

i. e. n = 2. In the same paper Jahoda reports that the temperature coefficient of the limiting current at low temperatures is too large to be explained solely as a function of the change of the diffusion constant of formaldehyde. It was also assumed that the behaviour mentioned could be explained if the rate of the electrode reaction was controlled by the depolymerisation of formaldehyde polymers, *e. g.* in accordance with reaction (A). However, Jahoda's assumption is not in agreement with the findings of Auerbach and Barschall [1] and Wadano *et al.*[2] Later Winkel and Proske [9] introduced the idea that only the dehydrated formaldehyde was able to take part in the electrochemical reaction at the electrode surface, and that the equilibrium corresponding to reaction (E) controlls the limiting current. This approch has subsequently been applied by Veselý and Brdička [10] and Bieber and Trümpler [11]. Their work has demonstrated that no real concentration polarisation occurs at the electrode surface; the limiting current is determined by the rate of dehydration of methylene glycol when the formaldehyde concentration at the electrode surface decreases in consequence of the electrode reaction. According to Bieber and Trümpler [11], the limiting current is highly dependent on pH and temperature. In buffered solutions the relationship between limiting current and formaldehyde concentration was found to be linear; in unbuffered solutions this was not the

case. Veselý and Brdička [10] found that reaction (E) is subject to acid — base catalysis as defined by Brönsted, *i. e.* the current is not only dependent on pH but also on the activities of the acids and bases present.

Briefly, we have the following picture of the electrode reaction: When the equilibrium between formaldehyde and methylene glycol is disturbed by the reduction at the electrode surface, the aldehyde deficiency is compensated by dehydration of the glycol. If the rate of dehydration is small in comparison with the diffusion of formaldehyde, the latter rate determines the limiting current. If the rate of dehydration is high, the current is still determined by a rate of diffusion, but in this case the diffusion is that of methylene glycol. However, if the rates of dehydration and diffusion are of the same order, both are of importance.

In a simplified mathematical treatment on the polarography of formaldehyde, Veselý and Brdička [10] applied a method introduced by Wiesner [12] for similar electrode processes. This treatment is based on the statistical "half mean path" which can be traversed by a molecule between dehydration and hydration. In accordance with this, the limiting current can be expressed as follows:

$$\bar{i}_k = \frac{0.573 \sqrt{t_1 k_2 k_h}}{1 + 0.573 \sqrt{t_1 k_2 k_h}} \cdot \bar{i}_d^* \qquad (2)$$

where $\bar{i}_k$ = limiting current, "catalytic current", t_1 = drop time, k_2 = the rate constant of the dehydration of the methylene glycol under the conditions occurring and k_h = the equilibrium constant of reaction (E). $\bar{i}_d^*$ is the theoretical diffusion current, calculated from eq. (1), assuming that concentration polarisation of the analytical amount of formaldehyde is the current-determining step.

When considering the decrease in methylene glycol concentration at high pH due to a dissociation reaction in accordance with (D), the following equation was given:

$$\bar{i}_k = \frac{b \sqrt{a_{H_3O^+}}}{a_{H_3O^+} + K_a + b \sqrt{a_{H_3O^+}} + b K_a / \sqrt{a_{H_3O^+}}} \bar{i}_d^* \qquad (3)$$

where $b = 0.573 \sqrt{t_1 k_2 k_h}$, K_a = dissociation constant of reaction (D).

Veselý and Brdička used eq. (3) to calculate K_a from experimental data, and found a good agreement with earlier mentioned values.

However, the mathematical treatment reviewed above involves several approximations: a) The non-linearity of the concentration gradient and b) the change of this gradient due to the growth of the mercury drop are neglected. c) The assumption on "half the mean path" is not significant from the theoretical point of view.

In an investigation concerning the catalytic currents in the polarography of some organic acids, *e. g.* pyruvic acid, Koutecký and Brdička [13] have given a mathematical treatment not involving the approximations a) and c). If we apply this method of treatment to the polarography of formaldehyde we get:

$$\bar{i}_k = \frac{0.81 \sqrt{t_1 k_2 k_h}}{1 + 0.81 \sqrt{t_1 k_2 k_h}} \bar{i}_d^* \qquad (4)$$

This is valid if the electrode is regarded as a steady sphere with the same area as the mean area of the dropping electrode. The very small correction for the curved surface of the electrode is, as usual, neglected in this case as well. When the theories are tested on experimental data, eq. (2) and (4) are identical, as the difference in the constants 0.57 and 0.81 only changes the numerical value of the parameter $k_2 \cdot k_h$.

During 1953 rigorous mathematical solutions, valid for several kinds of catalytic currents were given by Koutecký [14-17]. A mathematical technique was used that originated from Mejman [18]. However, in the case of formaldehyde only a simplified solution was given (Koutecký [17]), valid under the same conditions as needed for eq. (2) and (4), *i. e.* when the gradient layer is very close to the electrode surface. The equation derived is

$$\bar{i}_k = \frac{0.87 \sqrt{t_1 k_2 k_h}}{1 + 0.87 \sqrt{t_1 k_2 k_h}} \bar{i}_d^* \tag{5}$$

In the following a rigorous treatment will be given, avoiding the approximations a), b) and c). The mathematical technique is based on the Mejman-Koutecký method.

THEORETICAL

When considering the influence of the growth of an electrode drop on the concentration gradient in the solution surrounding the drop, we get, in accordance with Ilkovič [7], the following equation:

$$\left(\frac{\partial C_{HCHO}}{\partial t}\right)_{diffusion} = D \frac{\partial^2 C_{HCHO}}{\partial x^2} + \frac{2x}{3t} \frac{\partial C_{HCHO}}{\partial x} \tag{6a}$$

where x is the distance from the electrode surface.
Since

$$\left(\frac{\partial C_{HCHO}}{\partial t}\right)_{catalysis} = k_2 C_{HO \cdot CH_2 \cdot OH} - k_1 C_{HCHO} \tag{6b}$$

we obtain

$$\frac{\partial C_{HCHO}}{\partial t} = D \frac{\partial^2 C_{HCHO}}{\partial x^2} + \frac{2x}{3t} \frac{\partial C_{HCHO}}{\partial x} + k_2 C_{HO \cdot CH_2 \cdot OH} - k_1 C_{HCHO} \tag{7a}$$

and correspondingly:

$$\frac{\partial C_{HO \cdot CH_2 \cdot OH}}{\partial t} = D \frac{\partial^2 C_{HO \cdot CH_2 \cdot OH}}{\partial x^2} + \frac{2x}{3t} \frac{\partial C_{HO \cdot CH_2 \cdot OH}}{\partial x} - k_2 C_{HO \cdot CH_2 \cdot OH} + k_1 C_{HCHO} \tag{7b}$$

where k_2 is the dehydration and k_1 the hydration rate constant of reaction (E) and $D \sim D_{HCHO} \sim D_{HO \cdot CH_2 \cdot OH}$ (this will be discussed later).

Initial conditions: $t = 0$, $x > 0$: $C_{HO \cdot CH_2 \cdot OH} = C^*_{HO \cdot CH_2 \cdot OH}$, $C_{HCHO} = C^*_{HCHO}$.
Boundary conditions: $t > 0, x = 0$: $C_{HCHO} = 0$, $(\partial C_{HO \cdot CH_2 \cdot OH}/\partial s) = 0$. (No flux of $HO \cdot CH_2 \cdot OH$ at the electrode surface.)

Introducing:

$$\psi = C_{HO} \cdot CH_2 \cdot OH + C_{HCHO},\ \varphi = C_{HO} \cdot CH_2 \cdot OH - \sigma \cdot C_{HCHO},\ k_2 = \varrho,\ k_1/k_2 = 1/k_h = \sigma \quad (8)$$

and adding eq. (7a) and (7b), then multiplying eq. (7a) by σ and subtracting from eq. (7b) will give the respective equations:

$$\frac{\partial \psi}{\partial t} = D \frac{\partial^2 \psi}{\partial x^2} + \frac{2x}{3t} \frac{\partial \psi}{\partial x} \quad (9a)$$

$$\frac{\partial \varphi}{\partial t} = D \frac{\partial^2 \varphi}{\partial x^2} + \frac{2x}{3t} \frac{\partial \varphi}{\partial x} - \varrho\,(1 + \sigma) \cdot \varphi \quad (9b)$$

Substituting: $l = \varrho \cdot (1 + \sigma)$, $s = x/\sqrt{\frac{12}{7} D t}$ and $\chi = l\ t$ (10)

we have from eq. (9a) and (9b):

$$\frac{\partial^2 \psi}{\partial s^2} + 2s \frac{\partial \psi}{\partial s} - \frac{12}{7} \cdot \chi \frac{\partial \psi}{\partial \chi} = 0 \quad (11a)$$

$$\frac{\partial^2 \varphi}{\partial s^2} + 2s \frac{\partial \varphi}{\partial s} - \frac{12}{7} \chi \frac{\partial \varphi}{\partial \chi} = \frac{12}{7} \chi \varphi \quad (11b)$$

Initial conditions: $s \to \infty$, $\varphi = 0$, $\psi = \psi^* = C^*_{HO} \cdot CH_2 \cdot OH + C^*_{HCHO}$, *i. e.* the analytical formaldehyde concentration of the solution.

Boundary conditions: $s = 0$, $\varphi = \psi$, $(\partial \varphi/\partial s) = -\sigma\,(\partial \psi/\partial s)$;

Eq. (11b) can be transformed into an equation of the (11a) type by means of the substitution $\varphi = \Phi \cdot \exp(-\chi)$. It is assumed that the (11a) type has a solution in the form of a power series:

$$\psi = \sum_i \psi_i(s)\, \chi^i, \text{ and } \varphi = e^{-\chi} \sum_i \Phi_i(s)\, \chi^i \quad (12)$$

where ψ_i (and Φ_i) is determined by the equation:

$$\frac{d^2\psi_i}{ds^2} + 2s \frac{d\psi_i}{ds} - 2\alpha_i \cdot \psi_i = 0 \quad (13)$$

If we assume that

$$K\alpha = \sum_{j=0}^{\infty} a_j^{\alpha} \cdot s^j,\ L\alpha = \sum_{j=0}^{\infty} c_j^{\alpha} \cdot s^{j+1} \quad (14)$$

are linear, independent solutions of eq. (13), and $a_0^{\alpha} = c_0^{\alpha} = 1$.

$a_{j+2}^{\alpha}/a_j^{\alpha} = 2\,(\alpha - j)/(j + 1)(j + 2)$, $c_{j+2}^{\alpha}/c_j^{\alpha} = 2\,(\alpha - j - 1)/(j + 2)(j + 3)$, and from this: $\lim_{s\to 0} K\alpha = 1$, $\lim_{s\to 0} L\alpha = 0$, $\lim_{s\to 0} K'\alpha = 0$ and $\lim_{s\to 0} L'\alpha = 1$.

For this the following recurrence equation is obtained:

$$p\alpha\, p\alpha{+}1 = 2\,(\alpha + 1);\ p\alpha = \lim_{s\to\infty} K\alpha\,(s) \,/\, L\alpha\,(s) \quad (15)$$

($p\alpha$ can also be expressed as a Γ-function, *e. g.* for $\alpha = 6/7$ we have

$$p_{6\,i/7} = 2\,\Gamma(6\ i/14 + 1) \,/\, \Gamma(6\ i/14 + 1/2)).$$

From this, and with regard to the initial conditions, we get the solutions of eqs. (11a) and (11b):

$$\psi = \sum_{i=0}^{\infty} a_i\,(K\alpha i - p\alpha i \cdot L\alpha i) \cdot \chi^i + \psi^* p_0 L_0 \quad (16a)$$

$$\varphi = e^{-\chi} \sum_{i=0}^{\infty} b_i\,(K\alpha i - p\alpha i \cdot L\alpha i) \cdot \chi^i \quad (16b)$$

(The initial conditions are fullfilled since $\lim_{s\to\infty}(K_{\alpha i} - p_{\alpha i}\cdot L_{\alpha i}) = 0$ and $\lim_{s\to\infty} p_0 \cdot L_0 = 1$, as found when solving eq. (13) for L_0.)

Remembering that $L_{\alpha i}(0) = 0$ and $K_{\alpha i}(0) = 1$, we get from the boundary conditions:

$$\varphi_{s=0} = e^{-\chi}\sum_{i=0}^{\infty} b_i \cdot \chi^i, \quad \psi_{s=0} = \sum_{i=0}^{\infty} a_i \cdot \chi^i \tag{17}$$

i. e. $\varphi = \psi$ when $s = 0$ gives:

$$\sum_{i=0}^{\infty} b_i \cdot \chi^i = e^{\chi}\sum_{i=0}^{\infty} a_i \cdot \chi^i = \sum_{i=0}^{\infty}\sum_{j=0}^{i} \frac{a_j}{(i-j)!}\,\chi^i \tag{18}$$

The boundary condition $(\partial\varphi / \partial s) = -\sigma(\partial\psi / \partial s)$ when $s = 0$, and

$$\begin{aligned} \frac{\partial\psi}{\partial s} &= \sum_{i=0}^{\infty} a_i (K'_{\alpha i} - p_{\alpha i} L'_{\alpha i})\chi^i + \psi^* p_0 L_0' \\ \frac{\partial\varphi}{\partial s} &= e^{-\chi}\sum_{i=0}^{\infty} b_i (K'_{\alpha i} - p_{\alpha i} L'_{\alpha i})\chi^i \end{aligned} \tag{19}$$

since $K'_{\alpha i}(0) = 0$ and $L'_{\alpha i}(0) = 1$:

$$\begin{aligned} \left(\frac{\partial\psi}{\partial s}\right)_{s=0} &= -\sum_{i=0}^{\infty} a_i p_{\alpha i}\chi^i + \psi^* p_0 \\ \left(\frac{\partial\varphi}{\partial s}\right)_{s=0} &= -e^{-\chi}\sum_{i=0}^{\infty} b_i p_{\alpha i}\chi^i \end{aligned} \tag{20}$$

Then we have:

$$\frac{1}{\sigma}\sum_{i=0}^{\infty} b_i p_{\alpha i}\chi^i = \psi^* p_0 \sum_{i=0}^{\infty}\frac{\chi^i}{i!} - \sum_{i=0}^{\infty}\sum_{j=0}^{i}\frac{a_j p_{\alpha j}}{(i-j)!}\chi^i$$

and for the i:th term:

$$\frac{1}{\sigma} b_i p_{\alpha i} = \psi^*\frac{p_0}{i!} - \sum_{j=0}^{i-1}\frac{a_j p_{\alpha j}}{(i-j)!} - a_i p_{\alpha i} \tag{21}$$

Similarly we have:

$$b_i = \sum_{j=0}^{i-1}\frac{a_j}{(i-j)!} + a_i \tag{22}$$

From eqs. (21) and (22) we have:

$$a_i p_{\alpha i} = \frac{\psi^* p_0\sigma}{(1+\sigma)\,i!} - \sum_{j=0}^{i-1}\frac{a_j p_{\alpha j}}{(i-j)!}\left(1 + \frac{p_{\alpha i}/p_{\alpha j} - 1}{1+\sigma}\right) \tag{23}$$

Or, when $\sigma \gg 1$:

$$a_i p_{\alpha i} = \frac{\psi^* p_0}{i!} - \sum_{j=0}^{i-1}\frac{a_j p_{\alpha j}}{(i-j)!}\left(1 + \frac{p_{\alpha i}/p_{\alpha j} - 1}{\sigma}\right) \tag{24}$$

The equation

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} + \frac{2x}{3t} \frac{\partial c}{\partial x}$$

and the initial and boundary conditions $x > 0$, $t = 0$: $C = C^*$, $x = 0$, $t > 0$: $C = 0$ correspond to the conditions of concentration polarisation. As has been shown by Koutecký *et al.*[19] we obtain from this:

$$i_d = - C^* \, p_0 \, n \, F \, q \, D \left(\frac{\partial s}{\partial x}\right)_{x=0}$$

(q = the average area of the mercury drop.) Since in accordance with Ilkovič [7]

$$i_k = - n \, F \, q \, D \left(\frac{\partial C_{HCHO}}{\partial x}\right)_{x=0} \quad \text{we have:}$$
$$i_k / i_d = \Omega = (\partial C_{HCHO} / \partial s)_{s=0} / p_0 \cdot C^* \tag{25}$$

In accordance with earlier parts of the paper:

$$\psi = C_{HO} \cdot CH_2 \cdot OH + C_{HCHO}, \quad (\partial C_{HO} \cdot CH_2 \cdot OH / \partial s)_{s=0} = 0$$

and we have: $$\Omega = (\partial \psi / \partial s)_{s=0} / p_0 \cdot C^*_{HCHO} \tag{26}$$

Since $\psi^* = C^*_{HO} \cdot CH_2 \cdot OH + C^*_{HCHO}$ and $C^*_{HO} \cdot CH_2 \cdot OH = \sigma \cdot C^*_{HCHO}$

(chemical equilibrium) we obtain:

$$\Omega = 1 + \sigma - \sum_{i=0}^{\infty} \frac{a_i \, p_{ai}}{p_0 \, C^*_{HCHO}} \chi^i \tag{27}$$

Or, on introducing the symbol

$$\varepsilon_i = a_i \, p_{ai} / p_0 \, C^*_{HCHO} \tag{28}$$

$$\Omega = 1 + \sigma - \sum_{i=0}^{\infty} \varepsilon_i \chi^i \tag{29}$$

From this, by means of eq. (23):

$$\varepsilon_i = \frac{\sigma}{i!} - \sum_{j=0}^{i-1} \frac{\varepsilon_j}{(i-j)!} \left(1 + \frac{p_{ai} / p_{aj} - 1}{1 + \sigma}\right) \tag{30}$$

Or, when $\sigma \gg 1$:

$$\varepsilon_i = \frac{\sigma}{i!} - \sum_{j=0}^{i-1} \frac{\varepsilon_j}{(i-j)!} \left(1 + \frac{p_{ai} / p_{aj} - 1}{\sigma}\right) \tag{31}$$

(Since $\Omega = 1$ when $\chi = 0$ we find $\varepsilon_0 = \sigma$.)

However, if we start from the φ-function, we obtain:

$$\Omega = - (\partial \varphi / \partial s)_{s=0} / \sigma \, p_0 \, C^*_{HCHO} \tag{32}$$

and if we write $\nu_i = b_i \, p_{ai} / p_0 \, C^*_{HCHO}$ we obtain for $\sigma \gg 1$:

$$\nu_i = \frac{\sigma}{i!} - \sum_{j=0}^{i-1} \frac{\varepsilon_j}{(i-j)!} (1 - p_{ai} / p_{aj}) \tag{33}$$

where ε_j is defined in accordance with eq. (31),

$$i.\,e. \qquad \Omega = \frac{1}{\sigma} e^{-\chi} \sum_{i=0}^{\infty} \nu_i \chi^i \tag{34}$$

For small values of ϱ and large values of σ a solution can be obtained as follows: If these conditions are valid, $C_{HO} \cdot c_{H_3} \cdot OH$ can be regarded as being constant, *i. e.* $C^*_{HO} \cdot c_{H_3} \cdot OH$, and we get:

$$\frac{\partial C_{HCHO}}{\partial t} = D \frac{\partial^2 C_{HCHO}}{\partial x^2} + \frac{2x}{3t} \frac{\partial C_{HCHO}}{\partial x} + \varrho \sigma \left(\frac{C^*_{HO} \cdot c_{H_3} \cdot OH}{\sigma} - C_{HCHO} \right) \tag{35}$$

Initial and boundary conditions: $t = 0, x > 0$: $C_{HCHO} = C^*_{HCHO}$; $t > 0, x = 0$: $C_{HCHO} = 0$.

Introducing $C^*_{HO} \cdot c_{H_3} \cdot OH / \sigma - C_{HCHO} = \tau$, $\varrho \sigma = l$, $(l = \varrho (1 + \sigma) \sim \varrho \sigma, \sigma \gg 1.)$

and $s = x / \sqrt{\frac{12}{7} D t}$, $\chi = l t$:

$$\frac{\partial^2 \tau}{\partial s^2} + 2s \frac{\partial \tau}{\partial s} - \frac{12}{7} \chi \frac{\partial \tau}{\partial \chi} = \frac{12}{7} \chi \tau \tag{36}$$

Initial and boundary conditions: $\chi = 0$, $s \to \infty$: $\tau = 0$; $\chi > 0$, $s = 0$, $\tau = \tau^* = C^*_{HO} \cdot c_{H_3} \cdot OH / \sigma$.

In accordance with earlier parts of the paper:

$$\tau = e^{-\chi} \sum_{i=0}^{\infty} a_i \, (K_{\alpha i} - p_{\alpha i} L_{\alpha i}) \chi^i \tag{37}$$

Here a_i has to be chosen so that the following boundary condition is satisfied:

$$\tau_{s=0} = e^{-\chi} \sum_{i=0}^{\infty} a_i \chi^i = \tau^*$$

Since $K_{\alpha i}(0) = 1$, $L_{\alpha i}(0) = 0$:

$$a_i \chi^i = e^{\chi} \tau^* = \tau^* \sum_{i=0}^{\infty} \frac{\chi^i}{i!}$$

and

$$a_i = \tau^* \frac{1}{i!} \tag{38}$$

$K'_{\alpha i}(0) = 0$ and $L'_{\alpha i}(0) = 1$ gives:

$$\left(\frac{\partial \tau}{\partial s} \right)_{s=0} = - \tau^* e^{-\chi} \sum_{i=0}^{\infty} p_{\alpha i} \frac{\chi^i}{i!}$$

and

$$\Omega = e^{-\chi} \sum_{i=0}^{\infty} \frac{p_{\alpha i}}{p_0} \frac{\chi^i}{i!} \tag{39}$$

Recapitulating, the results of the three different treatments given above are:

$$\text{I. } \Omega = 1 + \sigma - \sum_{i=0}^{\infty} \varepsilon_i \chi^i$$

$$\text{a) } \varepsilon_i = \frac{\sigma}{i!} - \sum_{j=0}^{i-1} \frac{\varepsilon_j}{(i-j)!}\left(1 + \frac{p_{ai}/p_{aj} - 1}{1+\sigma}\right); \text{ general solution.}$$

$$\text{b) } \varepsilon_i = \frac{\sigma}{i!} - \sum_{j=0}^{i-1} \frac{\varepsilon_j}{(i-j)!}\left(1 + \frac{p_{ai}/p_{aj} - 1}{\sigma}\right); \ \sigma \gg 1.$$

$$\text{II. } \Omega = \frac{1}{\sigma} e^{-\chi} \sum_{i=0}^{\infty} \nu_i \chi^i$$

$$\nu_i = \frac{\sigma}{i!} - \sum_{j=0}^{i-1} \frac{\varepsilon_j}{(i-j)!}(1 - p_{ai}/p_{aj}); \ \sigma \gg 1$$

$$\text{III. } \Omega = e^{-\chi} \sum_{i=0}^{\infty} \frac{p_{ai}}{p_0} \frac{\chi^i}{i!} \text{ ; } \varrho \text{ comparatively small, } \sigma \gg 1.$$

For numerical calculations we need p_{ai} and p_{ai}/p_{aj}. If we compare eqs. (11a) and (13) we find $\alpha = 6/7$. By means of the earlier mentioned Γ-function and eq. (15) such calculations can be made. For ε_i, $\sigma \gg 1$, we obtain the following results: $\varepsilon_0 = \sigma$, $\varepsilon_1 = -0.500$, $\varepsilon_2 = 0.0585$, $\varepsilon_3 = -0.0112$, $\varepsilon_4 = 0.00320$, $\varepsilon_5 = -0.00101$, $\varepsilon_6 = 0.000226$ and $\varepsilon_7 = -0.0000491$. Since $\varepsilon_0 = \sigma$ and $\varepsilon_{i \neq 0} \ll \sigma$ we find that $\nu_i \sim \sigma \, p_{ai}/p_0 \, i!$; we here have the same factor as in solution III.

$p_{ai}/p_0 \, i!$ has, for other purposes, been calculated by Koutecký [16]. In that paper Koutecký has treated electrode reactions of the type $A \overset{el}{\rightleftharpoons} B$, *i. e.* the reduced compound B is reoxidized to A by an oxidizing agent, which is present in such an excess in the solution that its concentration can be regarded as constant even at the electrode surface. The solution obtained is the same as III, and it can easily be found that this problem and the reduction of formaldehyde under the conditions of III can be regarded as identical processes, even though Koutecký's mathematical treatment was somewhat different. When assuming $\sigma \gg 1$ ($\sigma \sim 10^4$, as will be discussed later), calculations on Ω as a function of χ from I—III give the same results for the χ-values in the range of practical interest; however, the slow convergence of the series makes them less suitable at high χ-values. Calculations under this condition will be discussed later.

Since the function i_k/i_d relates to the change in current during the life of the mercury drop, and in polarography the average current is measured, we have to find

$$\overline{\Omega} = \overline{i_k} / \overline{i_d}$$

where $\overline{i_k}$ and $\overline{i_d}$ are the average currents. The average current is defined by the equation

$$\overline{i} = \frac{1}{t_1} \int_0^{t_1} i \, dt$$

where t_1 is the drop time. (Ilkovič [7]). It is also found from Ilkovič that i_d = constant. $t^{1/6}$, and then we have:

$$\overline{\Omega} = \frac{\bar{i}_k}{\bar{i}_d} = \frac{\int_0^{t_1} \frac{i_k}{i_d} t^{1/6}\,dt}{\int_0^{t_1} t^{1/6}\,dt} \tag{40}$$

and

$$\overline{\Omega} = \frac{\bar{i}_k}{\bar{i}_d} = \frac{7}{6}\,\chi_1^{-\frac{7}{6}} \int_0^{\chi_1} \Omega\,\chi^{1/6}\,d\chi \tag{41}$$

$(\chi_1 = l \cdot t_1)$

The $\overline{\Omega}$ value related to III and the Ω function too, can be found in Koutecký [16], where they were calculated for the electrode reaction mentioned previously. In the same paper it was also shown that when $\chi_1 > 10$ the following empirical equation approximates a solution of the $\overline{\Omega}$ function:

$$\overline{\Omega} = 0.812\,\chi_1^{1/2} + 1.92\,\chi_1^{-7/6} \tag{42}$$

Then, when $\chi_1 \gg 1$, we get the asymptotic solution:

$$\overline{\Omega} = 0.81\sqrt{\chi_1} \tag{43}$$

For moderate and large χ_1, three different equations have earlier been derived — (2), (4) and (5) — suitable for calculations. Eq. (5) is an approximation of the expression:

$$\bar{i}_k / \bar{i}_{d\,HO\cdot CH_2\cdot OH} = \sum_{i=0}^{\infty} \omega_i \eta^i,\ \omega_i = \delta_i / (1 - 3\ i/7),$$

$$\eta = \sigma / \sqrt{\frac{12}{7} l\, t_1},\ \delta_0 = 1 \text{ and } \delta_{i+1}/\delta_i = -p_{-3\,(i+1)/7}.$$

or, for moderate values of $l\ t_1$:

$$\bar{i}_k / \bar{i}_{d\,HO\cdot CH_2\cdot OH} = \sum_{i=1}^{\infty} \beta_i \eta^{-i},\ \beta_i = \sqrt{\pi}\,\gamma_i / 2\,(1 + 3\,i/7),$$

$\gamma_1 = 1$ and $\gamma_{i+1}/\gamma_i = -p_{3\,i/7}$ ($\varrho\,\sigma \gg 1, \sigma \gg 1$. Koutecký [17])

From this we obtain $\beta_1 = 0.62$ and since $\eta^{-1} = \sqrt{\frac{12}{7} t_1 k_2 k_h} \cdot \frac{1}{\sigma}$:

$$\bar{i}_k / \bar{i}_{d\,HO\cdot CH_2\cdot OH} = 0.81\sqrt{t_1 k_2 k_h} \tag{44}$$

for moderate values of $k_2\ k_h$. The same result can be obtained from eq. (4) under the same conditions. Since $\chi_1 = k_2 t_1 / k_h$ and $k_h \bar{i}_{d\,HO\cdot CH_2\cdot OH} = \bar{i}_{d\,HCHO}$ when $\sigma \gg 1$, eq. (43) can be written as eq. (44).

When dealing with formaldehyde we have, as will be shown later in this paper, $\sigma \gg 1$. That is, the functions deduced above, which are valid for small and moderate values of χ_1, coincide asymptotically at $0.81 \sqrt{t_1 k_2 k_h}$ with the solutions for large values. From this we find that the two functions cover a large range of dehydration rates. For small and moderate values of $\sqrt{t_1 k_2 k_h}$ eq. (44) is found to be a better approximation than the function $0.87 \sqrt{t_1 k_2 k_h}$, which is obtained from eq. (5), and when considering the whole range of validity, eq. (4) is probably better than eq. (5), as regards the accuracy. However, it has to be remembered that these equations involve the approximation $D_{HCHO} \sim D_{HO \cdot CH_2 \cdot OH}$; this is not the case with the functions for small values of χ_1. (The diffusion of $HO \cdot CH_2 \cdot OH$ is negligible, as may be seen from comparisons between eqs. (6a), (6b) and (35) and the solutions corresponding to these equations.) Since the gradient layer at the electrode surface is thin (which can be found from the experimental results given later), the error introduced when neglecting the curvature of the electrode surface seems to be infinitesimal.

As was shown by *e. g.* Veselý and Brdička [10], a pure catalytic current is independent of the height of the mercury column between the level of the mercury in the reservoir and the tip of the capillary. A diffusion current, however, is proportional to the square root ot this height. This is the case since:

$$\bar{i}_d = \text{constant} \cdot m^{2/3} t_1^{1/6} \quad \text{and} \quad \bar{i}_k = \text{constant} \cdot m^{2/3} t_1^{2/3}$$

which easily can be derived from eq. (1) and (44).

An analysis of the dependence of the current upon the drop time can be used in order to investigate the electrode reaction; and in the case of formaldehyde we have:

A. $\bar{i} \cong \bar{i}_{d\,HCHO}$ $\bar{i}$ dependent of the drop time.

B. $\bar{i}_{d\,HCHO} \ll \bar{i} \ll \bar{i}_{d\,HO \cdot CH_2 \cdot OH}$ $\bar{i}$ independent of the drop time.

C. $\bar{i} \cong \bar{i}_{d\,HO \cdot CH_2 \cdot OH}$ $\bar{i}$ dependent of the drop time.

In the case A the limiting current is a function of ϱ, σ and t_1 as predicted by eqs. (39) and (41). Since

$$\lim_{\chi_1 \to 0} \bar{i}_k / \bar{i}_{d\,HCHO} = 1 \tag{45}$$

it is possible to determine σ from the effect of t_1 on the limiting current. For this purpose we can from experimental data plot the function:

$$\bar{i} / m^{2/3} t_1^{1/6} = f(t_1) \tag{46}$$

On extrapolating to $t_1 = 0$, *i. e.* $\chi_1 = 0$, we get, by means of eq. (1), $C_{o\,HCHO}$, and when the analytical concentration of formaldehyde is known, σ can be calculated. A better accuracy than that of the extrapolation can be obtained by the following method:

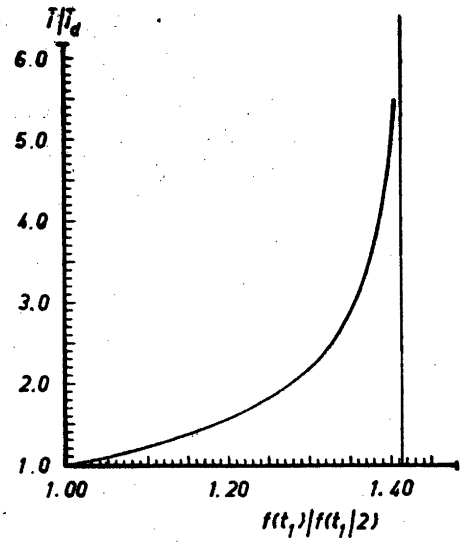

Fig. 1. $\bar{i}/\bar{i}_d$ *as a function of* $f(t_1)/f(t_1/2)$.

We have:

$$f(t_1)/f(t_1/2) = (\bar{i}_k/\bar{i}_d)_{t_1}/(\bar{i}_k/\bar{i}_d)_{t_1/2} = \bar{\Omega}(\chi_1)/\bar{\Omega}(\chi_1/2) = g(\chi_1)$$

$g(\chi_1)$ as being a function of χ_1 can be calculated from eqs. (39) and (41). Then $\bar{\Omega}(\chi_1) = (\bar{i}_k/\bar{i}_{d\,HCHO})_{t_1}$ is obtained from the equations mentioned above, *i. e.* a function $\bar{i}_k/\bar{i}_{d\,HCHO} = h(f(t_1)/f(t_1/2))$ can be deduced. This function is given in Fig. 1.

And from this $\bar{i}_{d\,HCHO}$, *i. e.* both ϱ and σ can be found. In case B the limiting current follows eq. (43), in the case C eq. (4) or at very high pH values eq. (3)*. *I. e.* $\sigma = 1/k_h$ and $\varrho = k_2$ can be calculated for the whole range A—C. From this discussion we find that both the equilibrium constant and the dehydration rate constants for the system methylene glycol-formaldehyde can be studied by means of polarography.

In accordance with reaction (F) hydroxyl ions are produced at the electrode surface during the polarographic reduction of formaldehyde. Since reaction (E) is acid-base catalysed, we can assume that in an unbuffered or slightly buffered solution no linear limiting current-formaldehyde concentration curve can be found. That this is the case in unbuffered solutions was shown by Bieber and Trümpler [11]. Since the hydroxyl ions change the composition and the catalytic properties of the solution surrounding the electrode drop, the change of cource depends on the degree of buffering, *i. e.* the more buffered the solution, the more linear the current-concentration curve. The mathematical analysis of a case involving a moderate degree of buffering seems to be very difficult; for unbuffered solutions a simple, approximate treatment can be given: From eqs. (1) and (43) we have:

$$\bar{i}/C_{HO}\cdot c_{H_2}\cdot c_{OH}\; m^{2/3}\, t_1^{1/6} = \text{constant}\cdot\sqrt{t_1 k_2 k_h} \qquad (47)$$

* as found from the previous treatment $b = 0.81\sqrt{t_1 k_2 k_h}$ is to be preferred.

The dehydration rate constant represents the sum of the products of the activity of the catalyst and the catalysing power constant:

$$k_2 = \sum_i k_i \, a_i \tag{48}$$

(Veselý and Brdička [10]). For unbuffered solutions we obtain a simplified solution to the problem if we only consider the OH^- concentration. And in such a case:

$$(\bar{i} \,/\, C_{HO} \cdot {}_{CH_2} \cdot {}_{OH} \, m^{2/3} \, t_1^{1/6})^2 = \text{constant} \cdot t_1 \; C_{o\,OH^-} \tag{49}$$

If the reaction layer is supposed to be very thin, $C_{o\,OH^-}$, the average concentration of hydroxyl ions in this layer, can be found from eq. (1), and

$$\bar{i} = \text{constant} \cdot (1 \,/\, m^{2/3} \; t_1^{7/6}) \; (\bar{i} \,/\, C_{HO} \cdot {}_{CH_2} \cdot {}_{OH})^2$$

or, when the capillary characteristics are constant:

$$\bar{i} = \text{constant} \cdot (\bar{i} \,/\, C_{HO} \cdot {}_{CH_2} \cdot {}_{OH})^2 \tag{50}$$

This simplified solution is applicable in the range of validity of eq. (43) to solutions which are unbuffered or have a very low degree of buffering. (In the latter case, however, a slight contribution from the buffer substance catalysis can be expected.) As has been shown by Bieber and Trümpler [11] the half wave potential of formaldehyde increases with increasing pH. In the case mentioned above more or less skew polarograms can be expected, since the increase in current means an increase in pH.

EXPERIMENTAL

The investigations have been carried out in the following apparatus: In a large beaker, containing 0.1 *M* KCl, a "polarographic cell" is immersed. The solution to be investigated is brought into this cell in such a quantity that the level is above that of the KCl solution. This is done in order to prevent any back flow into the cell through the glass filter disc which separates the cell solution from that of the beaker. The capillary enters the cell by means of a cork stopper, and nitrogen can be bubbled through the solution in the cell, in order to remove dissolved oxygen. The gases can escape through a water trap; the use of this device prevents air from entering the cell. The flow of mercury at the electrode is determined by means of a funnel-fitted capillary, which enters the cell through the stopper. The funnel can be turned into the line of the dropping mercury, and after the required time has elapsed, the funnel is swung clear of the drops. The mercury in the funnel is then sucked up into a flask connected to the capillary outside the cell. The flask is then removed and the mercury washed with distilled water, after which it is dried and weighed. The mercury of the electrode is delivered from a reservoir, connected to the capillary by means of a thick—walled rubber tubing, which has been boiled in NaOH and rigorously rinsed and dried. The reference electrode is a saturated calomel electrode, immersed in the KCl solution. The electrode device is thermostated by means of an infrared lamp outside the beaker, and a contact thermometer and a relay give an on-off regulation of the lamp. The beaker solution is kept in motion by a stirrer. One of the advantages of this thermostating device is that the flow of heat is cut off practically at the same time as the current. By adjusting the distance between the lamp and the beaker the on-off periods can be given a suitable length. The temperature can easily be controlled within 0.03° C. Since the purity of the mercury used is of greatest importance, the mercury was distilled in a vacuum apparatus.

All chemicals in the investigation were of A. R. quality, except the formaldehyde, which was a Merck product of high purity, only containing traces of methanol and formic acid. ($<10^{-4}$ *M* in a solution containing ~380 g/l formaldehyde).

Table 1. Results of the polarographic measurements on formaldehyde.

Supporting electrolyte	$C_{(HCHO)a}$ mmole/l	u cm	$\bar{i}$ μA	t sec	m mg/sec	$\frac{\bar{i}}{m^{2/3} t^{1/6} C}$	$\frac{\bar{i}}{m^{2/3} t^{2/3} C}$
0.0025 *M* KH_2PO_4 + 0.0025 *M* Na_2HPO_4 pH 6.86, 20° C	12.7	11.1 11.1 11.1					
		11.1	0.333	2.07	4.55	0.00846	0.00587
		11.0 10.9 11.0					
		11.0	0.330	3.00	3.05	0.0103	0.00594
		10.8 10.9 10.9					
		10.9	0.327	4.01	2.34	0.0116	0.00582
		10.8 10.7 10.8					
		10.8	0.324	4.93	1.89	0.0128	0.00577
pH 6.86	25.4	11.1 11.2 11.1					
		11.1	0.666	2.07	4.47	0.00852	0.00592
		11.0 11.1 11.0					
		11.0	0.660	3.04	3.09	0.0102	0.00585
		10.9 10.9 10.9					
		10.9	0.654	4.04	2.30	0.0117	0.00581
		10.8 10.8 10.7					
		10.8	0.648	5.02	1.86	0.0129	0.00575

Supporting electrolyte	$C_{(HCHO)_a}$ mmole/l	u cm	$\bar{i}$ μA	t sec	m mg/sec	$\frac{\bar{i}}{m^{2/3}t^{1/6}C}$	$\frac{\bar{i}}{m^{2/3}t^{2/3}C}$
0.0050 *M* KH_2PO_4 + 0.0050 *M* Na_2HPO_4 pH 6.86, 20° C	12.7	12.0 11.9 12.0					
		12.0	0.360	2.10	4.55	0.0099	0.00683
		11.8 11.7 11.8					
		11.8	0.354	2.94	3.06	0.0112	0.00656
		11.7 11.7 11.7					
		11.7	0.351	4.25	2.36	0.0136	0.00658
		11.6 11.6 11.5					
		11.6	0.354	5.88	1.74	0.0158	0.00652
pH 6.86	25.4	17.8 17.8 17.8					
		17.8	0.712	2.48	3.44	0.0106	0.00671
		17.7 17.7 17.7					
		17.7	0.710	2.82	3.07	0.0111	0.00662
		17.6 17.6 17.5					
		17.6	0.705	4.21	2.10	0.0135	0.00658
		17.4 17.4 17.4					
		17.4	0.695	5.82	1.45	0.0156	0.00646

Supporting electrolyte	$C_{(HCHO)_a}$ mmole/l	u cm	$\bar{i}$ μA	t sec	m mg/sec	$\bar{i}/m^{2/3}t^{1/6}C$	$\bar{i}/m^{2/3}t^{2/3}C$
0.010 *M* Na_2CO_3 pH 9.54, 20° C	6.35	12.2 12.2 12.2					
		12.2	0.448	2.75	2.82	0.0326	0.0197
		12.0 12.0 12.0					
		12.0	0.480	3.41	2.04	0.0382	0.0208
		11.9 11.9 11.9					
		11.9	0.437	4.80	1.45	0.0444	0.0202
pH 9.54	12.7	12.3 12.2 12.3					
		12.3	0.985	2.08	3.36	0.0305	0.0210
		12.1 12.1 12.0					
		12.1	0.968	3.11	2.27	0.0366	0.0208
		11.9 11.9 11.9					
		11.9	0.953	4.88	1.43	0.0455	0.0206
0.010 *M* Na_2CO_3 pH 10.44, 20° C	2.03	13.1 13.1 13.1					
		13.1	0.394	3.39	2.28	0.0920	0.0495
		13.0 13.1 13.1					
		13.1	0.394	3.82	2.04	0.0966	0.0494
		13.0 13.0 13.1					
		13.0	0.390	5.12	1.52	0.108	0.0490

Supporting electrolyte	$C_{(HCHO)a}$ mmole/l	u cm	$\bar{i}$ μA	t sec	m mg/sec	$\frac{\bar{i}}{m^{2/3}t^{1/6}C}$	$\frac{\bar{i}}{m^{2/3}t^{2/3}C}$
pH 10.46	4.07	19.7 19.6 19.7					
		19.7	0.789	2.78	2.82	0.0824	0.0494
		19.6 19.6 19.6					
		19.6	0.785	3.76	2.08	0.0945	0.0489
		19.6 19.5 19.5					
		19.5	0.780	5.11	1.52	0.109	0.0486
0.010 *M* Na_2CO_3 pH 10.83, 20° C	0.635	13.3 13.3 13.3					
		13.3	0.160	2.01	3.85	0.0915	0.0645
		13.4 13.4 13.4					
		13.4	0.161	4.10	1.95	0.129	0.0640
		13.3 13.4 13.3					
		13.3	0.160	5.26	1.48	0.147	0.0642
pH 10.81	1.27	10.7 10.6 10.7					
		10.7	0.321	2.06	3.87	0.0915	0.0648
		10.7 10.8 10.8					
		10.8	0.324	4.12	1.94	0.131	0.0645
		10.7 10.7 10.7					
		10.7	0.321	5.30	1.48	0.147	0.0642

Supporting electrolyte	$C_{(HCHO)a}$ mmole/l	u cm	$\bar{i}$ μA	t sec	m mg/sec	$\bar{i}/m^{2/3}t^{1/6}C$	$\bar{i}/m^{2/3}t^{2/3}C$
0.10 . *M* NaOH	0.127	10.1					
		10.1					
pH ~ 12.7, 20° C		10.0					
		10.1	0.202	2.81	2.72	0.690	0.410
		9.6					
		9.7					
		9.6					
		9.6	0.185	4.22	1.69	0.810	0.395
		8.8					
		8.8					
		8.7					
		8.8	0.177	5.85	1.32	0.880	0.380
pH ~ 12.7	0.353	13.5					
		13.4					
		13.5					
		13.5	0.540	2.78	2.60	0.689	0.412
		12.6					
		12.7					
		12.6					
		12.6	0.509	4.20	1.65	0.810	0.394
		12.0					
		12.0					
		12.0					
		12.0	0.481	5.88	1.26	0.870	0.358

The technique and chemicals described above were subsequently used in order to investigate the relationship between limiting current, flow of mercury and drop time at 20 ± 0.03° C for the solution compositions shown in Table 1. The formaldehyde concentration was determined on the stock solution by the method of Blank and Finkenheimer, and a result of 380 ± 2 g/l was obtained. The buffer substances of the supporting electrolytes were dissolved in a measuring flask, the required amount of formaldehyde added, and the solution diluted to a volume close to the final. By means of HCl or NaOH pH was adjusted. For the pH determinations the glass electrode differential technique described in the Swedish Patent 129833 was applied, and an accuracy of ± 0.01 pH obtained. The solutions prepared were kept at 20° C for 24 hours, in order to prevent any influence from reaction (A). Then the pH was rechecked (no changes found), the solutions made up to the final volume with water and poured into the cell. Since the second wave of dissolved oxygen interferes with the formaldehyde wave at low concentrations of the aldehyde, a removal of dissolved oxygen is necessary, especially when a high degree of accuracy is needed. This was achieved by bubbling nitrogen slowly through the solution in the cell; this also provides valuable stirring during the period of temperature stabili-

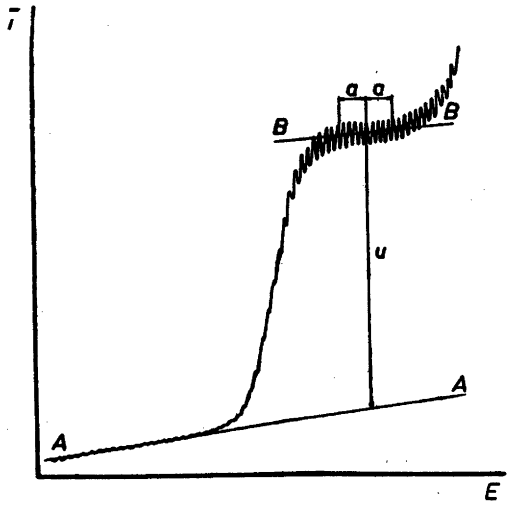

Fig. 2. Polarogram of formaldehyde, $C_{(HCHO)a} = 1.27 \times 10^{-3}$, 20° C, supporting electrolyte: 0.010 M Na_2CO_3, pH 10.81.

sation. Here, of course, we encounter the question of whether the formaldehyde concentration is disturbed during the process, *i. e.* whether any formaldehyde is removed by the nitrogen. However, it was found that during the time of bubbling used in this case, 30 minutes, no change could be found in any of the solutions investigated. It might be assumed that a rapid flow of nitrogen and very long bubbling times have to be avoided, especially at high pH values, since the rate of dehydration is then high. The capillary tubes were carefully cleaned, in order to make the drop cycle completely reproducible. A suitable method for cleaning is to suck concentrated nitric acid through the capillary tube for 10 minutes, then distilled water for 10 minutes, and finally methanol. The suction has to be maintained until the capillary is completely dry.

When evaluating the polarograms the method shown in Fig. 2 has been applied. With the small currents occurring in this case it is necessary to make a correction for the condenser current. The usual method for such a correction has been applied, *i. e.* the line A—A along the average current curve is drawn. The limiting current is then determined by the vertical distance u between the line A—A and the point of inflexion between formaldehyde and supporting electrolyte on the current-voltage curve. (This method has often been used, *e. g.* by Brdička [20].) The point of inflexion is found by drawing the mean current curve and the line B—B, and bisecting the part of the line that lies on the curve. The drop times and the flows of mercury were determined at the potential corresponding to the point of inflexion. The polarograms were recorded by means of a LKB type 3 266 polarograph and a Leeds & Northrup Speedomax G recorder. The rate of voltage increase was 0.1 V/min. As a secondary control a Radiometer type PO3g polarograph was used.

RESULTS

The results of the measurements are given in Table 1. The u values of three measurements for each composition of the solution are to be found in the table, the average of these values was used in the further treatments. The treatments involve the calculation of the numerical value of the function

$$\bar{i} \,/\, C_{(HCHO)a} \cdot m^{2/3}\ t_1^{1/6} \qquad (51)$$

i. e. the function (46), but including the analytical amount of formaldehyde ($\sigma \gg 1$). For each composition of the supporting electrolyte measurements

were made on two different aldehyde concentrations; this was done in order to find whether the earlier mentioned influence of the hydroxyl ions produced at the electrode surface is of importance. If this is the case, differences in the functions (51) should be found. Such differences did not occur, as can be seen from Table 1. The influence of the concentration of the buffer in the supporting electrolyte, *i. e.* the acid — base catalysis, is exemplified by the experiments at pH 6.86. In the diagrams 3—5 the expression (51) has been plotted against drop time, and from these curves functions in accordance with eq. (46) and the following discussion have been calculated. The results are given in Table 2,

Table 2. $f(t)/f(t/2)$ from experimental data.

Supporting electrolyte	pH	$C_{(HCHO)a} \times 10^3$	f(5)/f(2.5)
0.0050 *M* KH_2PO_4 + 0.0050 *M* Na_2HPO_4	6.86	12.7 25.4	1.41
0.0025 *M* KH_2PO_4 + 0.0025 *M* Na_2HPO_4	6.86	12.7 25.4	1.41
0.010 *M* Na_2CO_3	9.54	6.35 12.7	1.42
0.010 *M* Na_2CO_3	10.44 10.46	2.03 4.07	1.41
0.010 *M* Na_2CO_3	10.83 10.81	0.635 1.27	1.43
0.10 *M* NaOH	~12.7	0.127 0.353	1.30

and are in agreement with the postulates in Fig. 1: at pH 9.5, 10.4 and 10.8 the limit 1.4 is obtained. This is not the case at pH 12.7, as might be expected from eq. (3). The results at pH 6.86 are not obviously different from the values at pH 9.5—10.8, *i. e.* the equilibrium constant cannot be determined with a high degree of accuracy. In order to find whether the difference is statistically significant, the following expression has been included in the tables; since this is very suitable for tests on eq. (44):

$$\bar{i} \,/\, C_{(HCHO)a} \cdot m^{2/3}\, t_1^{2/3}$$

Regression analysis applied to the group pH 9.5, 10.4 and 10.8 and the group pH 6.86 reveals a significant difference. (I. Sandelius, private communication.)

From a diffusion constant of 16×10^{-6} cm²/sec and the fact that $\bar{i} / \bar{i}_d > 3.5$ (Fig. 1) it can be postulated that $k_h < 3 \times 10^{-4}$, and the value 10^{-4} seems to be very probable when regarding the significant difference mentioned above.

From measurements on the temperature dependence of the formaldehyde limiting current, Bieber and Trümpler [11] found an "activation energy" for this

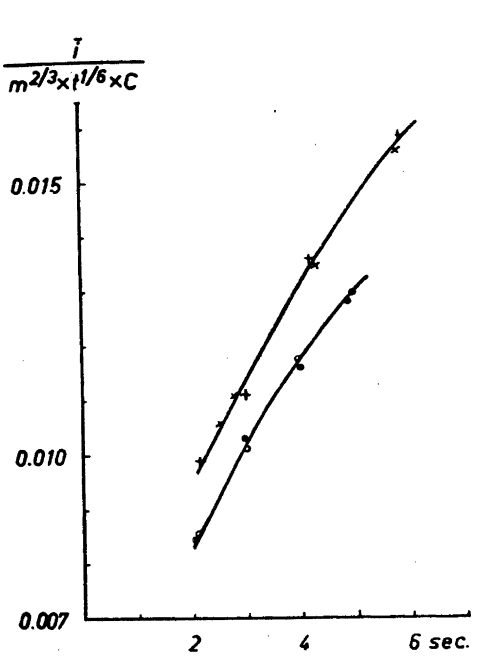

Fig. 3. Relation between function (51) and drop time, 20° C, pH 6.86; $C_{(HCHO)_a}$ = 12.7 × 10^{-3} (●), 25.4 × 10^{-3} (○), supporting electrolyte: 0.0025 M KH_2PO_4 + 0.0025 M Na_2HPO_4; $C_{(HCHO)_a}$ = 12.7 × 10^{-3} (+), 25.4 × 10^{-3} (×), supporting electrolyte: 0.0050 M KH_2PO_4 + 0.0050 M Na_2HPO_4.

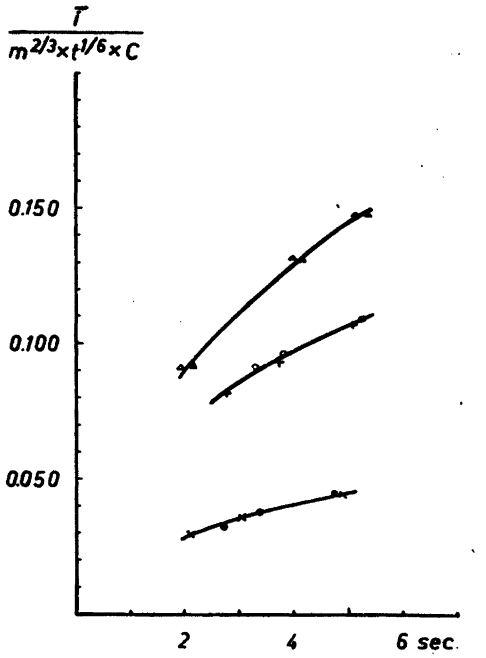

Fig. 4. Relation between function (51) and drop time, 20° C, 0.010 M Na_2CO_3; $C_{(HCHO)_a}$ = 6.35 × 10^{-3} (●), 12.7 × 10^{-3} (×), pH 9.54; $C_{(HCHO)_a}$ = 2.03 × 10^{-3} (+), pH 10.44; $C_{(HCHO)_a}$ = 4.07 × 10^{-3} (○), pH 10.46; $C_{(HCHO)_a}$ = 0.635 × 10^{-3} (△), pH 10.83; $C_{(HCHO)_a}$ = 1.27 × 10^{-3} (▲), pH 10.81.

current of the order 14 kcal. From spectrophotometrical data Bieber [6] deduced an activation energy of the dehydration of methylene glycol of 14.6 kcal. Since $k_1/k_2 = 1/k_h$ eq. (44) can be written:

$$\bar{i} / \bar{i}_d \text{ HO·CH}_2\text{·OH} = 0.81\ k_h \sqrt{t_1 k_1} \qquad (52)$$

From this we find that the hydration rate constant is only slightly dependent on the temperature. This means that measurements at other temperatures would not be more favourable when determining the equilibrium constant, since the change in this constant is chiefly dependent on the change in the dehydration rate. Measurements at 0° C and 50° C have confirmed this. Measurements at low pH values are also of no help, as can be found when examining the results from Bieber and Trümpler [21]; mixtures of methanol and water were also investigated in the present study, but no results of interest were obtained.

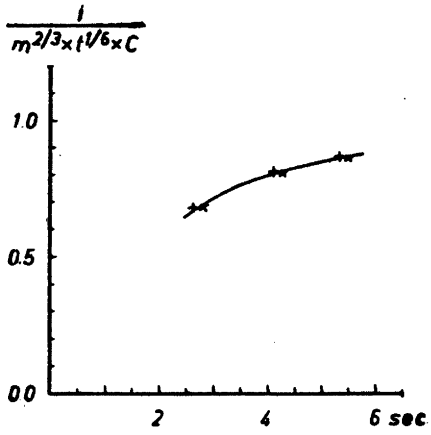

Fig. 5. Relation between function (51) and drop time, 20° C, 0.10 M NaOH, pH ~ 12.7; $C_{(HCHO)a} = 0.127 \times 10^{-3}$ (+), 0.353×10^{-3} (×).

In order to study the hydroxyl ion effect mentioned earlier, measurements were made on the following solutions at pH 9.20 and 20 ± 0.03° C:

$C_{borax} \times 10^3$: 0.5, 1.0, 2.5, 5.0 and 15.0: $C_{(HCHO)a} \times 10^2$: 0.5, 1.0, 2.5, 3.5 and 5.0, and at $C_{borax} = 0$: $C_{(HCHO)a} \times 10^2$: 1.0, 1.25, 1.50 and 1.75 (29 different solutions). $C_{HO \cdot CH_2 \cdot OH} = C_{(HCHO)a}$ since $\sigma \gg 1$.

The experimental results are given in Fig. 6, and in unbuffered or slightly buffered solutions the linear relationship required by eq. (50) is found. The

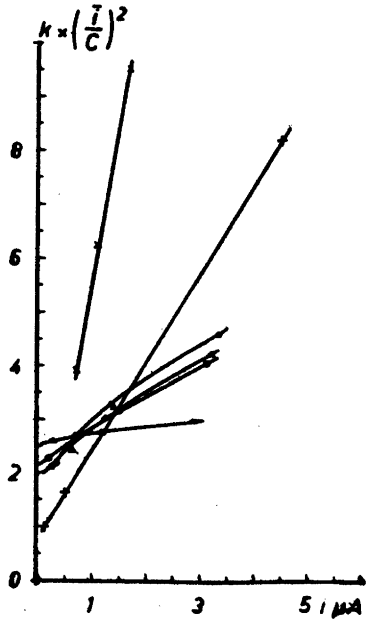

Fig. 6. Hydroxyl ion effect in unbuffered and slightly buffered solutions. $C_{borax} \times 10^3 = 0$ (×), 0.5 (+), 1.0 (●), 2.5 (Δ), 5.0 (○) and 15.0 (▲).

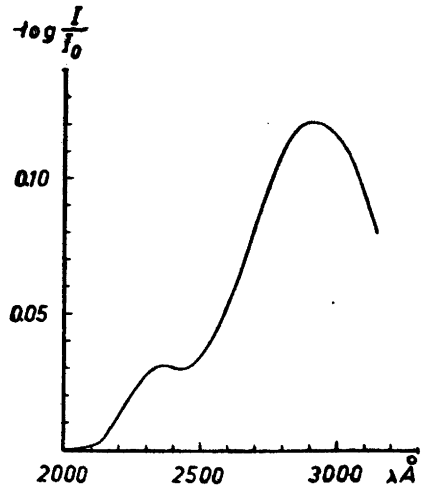

Fig. 7. Ultraviolet absorption curve of 4.75 M HCHO in 0.05 M KH_2PO_3 + KOH, pH 6.70, 50° C.

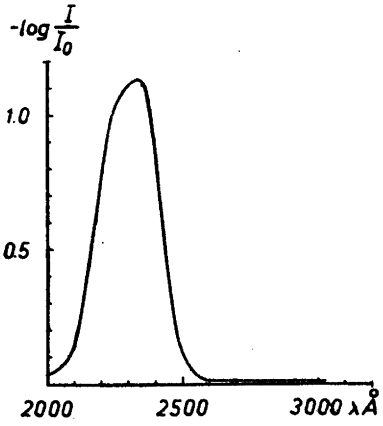

Fig. 8. An ultraviolet absorption curve of formic acid.

polarograms were also skew, in accordance with the earlier discussion. In the slightly buffered solutions the contribution of the buffer substance to the catalysis is shown by $(\bar{i}/C_{(HCHO)a})^2 \neq 0$ when $\bar{i} = 0$.

SPECTROPHOTOMETRICAL INVESTIGATIONS

Measurements in the ultraviolet range were made on formaldehyde in order to check Bieber's [6] result, and to find whether trioxymethylene glycol is of any importance at a temperature of 50° C. If such is the case, the extrapolation made by Bieber would be open to criticism.

The instrument used was a Hilger & Watt "Uvispec" spectrophotometer. The lid of the transmission cell housing was removed and replaced by another, with three holes. Through one of these connections hot air (~ 60° C) from a simple hair-drying device was blown into the housing, through another the air was allowed to escape through a long, curved rubber tubing, preventing any light leakage into the cell housing. Through the third hole a contact thermometer on-off regulates the air flow. The temperature of the cell solutions was measured by a thermo-couple, and the contact thermometer was adjusted so that the cell temperature was maintained at 50 ± 0.5° C. The cells, protected by air—tight rubber caps, were kept in the housing during 20 minutes, which time was found to be enough to give a constant temperature in the cell solutions.

By means of this device the wavelength interval 2 000—3 200 Å was investigated; cell length 2 cm, temperature 50° C, $C_{(HCHO)a}$: 1.27, 3.18, 3.81, 5.08 and 6.35. The solvent was 0.05 *M* KH_2PO_4 + KOH, pH 6.70. Fig. 7 is an example of the absorption curves obtained. The curves have absorption bands at 2 300 and 2 800 Å. As postulated by *e. g.* Bieber [6] the latter is the

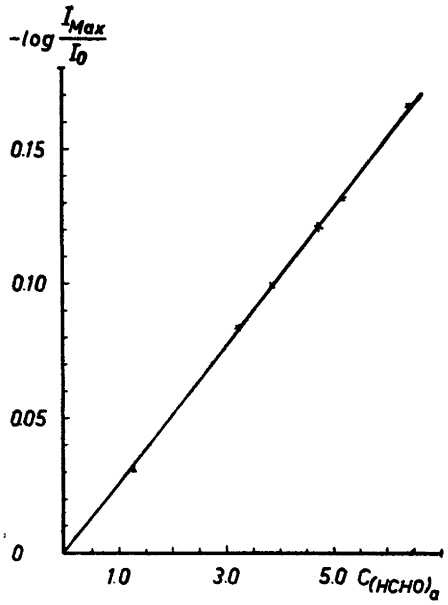

Fig. 9. Relation between maximum extinction and formaldehyde concentration, 0.05 M KH_2PO_4 + KOH, pH 6.70, 50° C.

formaldehyde band. The absorbtion in the 2 300 Å range is probably due to traces of formic acid present in the formaldehyde solution, the results of an investigation on a formic acid solution at the same pH and temperature makes this assumption very probable, as can be found from Fig. 8. Fig. 9 shows the relation between the maximum extinction in the 2 860 band and the formaldehyde concentration. If we use the extinction coefficient estimated by Bieber [6] (13.5), and calculate the equilibrium constant at 50° C, we get the value 0.96×10^{-3}. This is in good agreement with the result obtained if Bieber's results are extrapolated to the same temperature: 1.15×10^{-3}. As a linear relationship is found for maximum extinction and formaldehyde concentration, the trioxymethylene glycol from reaction (A) seems to be of no importance. In accordance with Auerbach and Barschall [1], the trioxymethylene glycol fraction increases with increasing concentration, *i. e.* the methylene glycol fraction decreases. Of course, this may be compensated by a change in the extinction coefficient, since at high concentrations there is a solvent mixture of water, methylene glycol and trioxymethylene glycol. However, the probability of such compensation seems to be very low. At 20° C the case mentioned above can not be neglected, *i. e.* the influence of trioxymethylene glycol. From measurements at 20° C, $C_{(HCHO)_a} = 6.35$, and from the equilibrium constant 10^{-4} we find $C_{HO \cdot CH_2 \cdot OH} / \Sigma\, C_{HCHO} = 0.6$. This result is in agreement with the figure expected from Walker [22]. However, the result cannot be assigned too much value, in view of the influence of the solution composition on the extinction coefficient and the low accuracy at the low absorption in question. Finally, solutions with $C_{(HCHO)_a} = 6.35$ have been studied at 50° C and pH 4.6, 6.7, 9.2 and 10.8. No pH dependence could be detected.

Table 3. Dehydration rate constants of methylene glycol.

Electrolyte	k_2 20° C
0.0050 *M* KH_2PO_4 + 0.0050 *M* Na_2HPO_4 pH 6.86	$\sim 1.9 \times 10^{-3}$
0.0025 *M* KH_2PO_4 + 0.0025 *M* Na_2HPO_4 pH 6.86	$\sim 1.7 \times 10^{-3}$
0.010 *M* Na_2CO_3	
pH 9.54	~ 0.3
pH 10.44	~ 1.8
pH 10.88	~ 2.8

From the above we see that nothing is in disagreement with Bieber's results. It has been shown that the extrapolation to 20° C is possible, since at 50° C no trioxymethylene glycol of practical importance seems to be present, even at high concentrations; Bieber's measurements have been made at a temperature above 50° C and a concentration below that used here. This means that the extrapolated values refer to dilute solutions. And we find that we have several reasons to accept Bieber's result:

$$k_h \simeq 10^{-4}$$

A comparison with the polarographic result, $k_h < 3 \times 10^{-4}$, and the result of the spectrophotometrical measurement at 20° C, makes the value given above very probable.

THE RATE OF DEHYDRATION OP METHYLENE GLYCOL

Since $\bar{i} / \bar{i}_d > 3.5$ even at pH 6.9, eq. (44) can be applied to the pH range 7—11 without appreciable error, and the order of the rates of dehydration of methylene glycol can be calculated.

From eq. (44) and $k_h \sim 10^{-4}$ we get:

$$k_{2\ 20°\,C} = 0.7 \times 10^{-3} \cdot \bar{i}^2 / C^2_{(HCHO)a} \cdot m^{4/3}\ t_1^{4/3} \qquad (53)$$

The reaction rate constants calculated from eq. (53) and based on the measurements presented in Table 1, can be found in Table 3.

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Studies on Glycosides and *Iso*propylidene Derivatives

Synthesis by Cation Exchanger Catalysis

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Methyl glycoside formation catalyzed by acid-regenerated cation exchange resins was studied by titrimetric, polarimetric, and paper chromatographic methods. Glucose, when reacted with methanol, gave reducing power and optical rotation curves of the same shape as those obtained on mineral acid catalysis. The sequence of the reaction products formed was examined. The main product was α-methyl D-glucopyranoside (yield above 80 %). Maltose and lactose gave rise only to traces of disaccharide glycosides, methyl glycosides of the component monosaccharides constituting the main products. Fructose was rapidly degraded by the acid resin.

Furthermore, the resin catalyzed formation of *iso*propylidene derivatives was studied. Fructose reacted readily with acetone at 20° C to 1,2-4,5-di*iso*propylidene D-fructopyranose. Glucose, at 20° C reacted only slowly, but with boiling acetone a mixture of 1,2-mono- and 1,2-5,6-di*iso*propylidene glucoses was obtained. In the presence of borate the acetonylation was impeded.

In many instances of acid catalysis protonated cation exchangers may replace the mineral acid. Hereby several advantages are achieved, including a gentle treatment of the reactants and a ready removal of the catalyst after finished reaction. Sussman [1], apparently first in this field, in 1946 reported on the formation and solvolysis of esters and acetals and the inversion of sucrose, under the influence of ion exchange catalysts. Later the kinetics of the exchanger catalyzed ester hydrolysis was studied by Haskell and Hammett [2], while the corresponding sucrose inversion was quantitatively elucidated by Bodamer and Kunin [3], and others. In 1952 Osman, Hobbs, and Walston [4] applied ion exchange resins to the preparation of methyl glucuronides from glucuronolactone, and in a similar way some methyl glycosides and sugar *iso*propylidene derivatives were synthesized by Cadotte, Smith, and Spriestersbach [5]. The relative efficiencies of ion exchangers and of sulfuric acid in some acid catalyzed sugar reactions were examined by Wadman [6]. It was found

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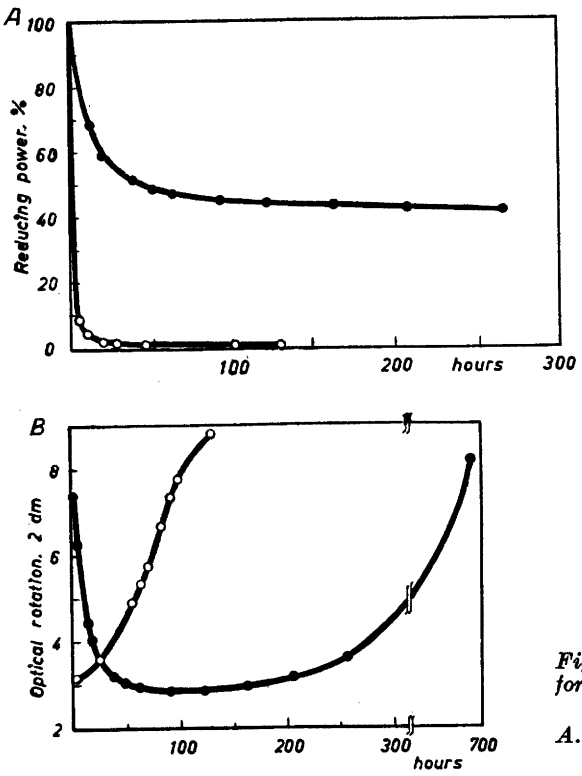

Fig. 1. Resin catalyzed methyl glucoside formation, at 20° C (full circles), and at 65° C (open circles).
A. Reducing power. B. Optical rotation.

that, on a molar basis, ion exchangers (sulfonic acid type) were less effective than the mineral acid.

The experiments reported in the present paper were performed in order to study, in some detail, the materials formed in the resin-catalyzed reactions of some sugars with methanol and acetone. Besides, the resin catalyzed acetonylation was applied to the isolation of sugars subsequent to an alkali-induced aldose isomerization.

Methyl glycosides

Some mono- and disaccharides were glycosidified with methanol using as catalysts acid-regenerated cation exchangers of the sulfonated polystyrene type.

Thus, glucose was reacted with methanol at 20° C, by shaking with methanol and catalyst, and at 65° C, by percolating a boiling glucose-methanol solution through a resin column in a closed system, utilizing the apparatus shown in Fig. 4. From time to time aliquots were withdrawn for analysis. Changes in reducing power and optical rotation are presented in Fig. 1 A and B.

The curves are similar to those obtained on mineral acid catalysis, as reported by Levene *et al.*[7] A difference in reaction velocity is apparent, however,

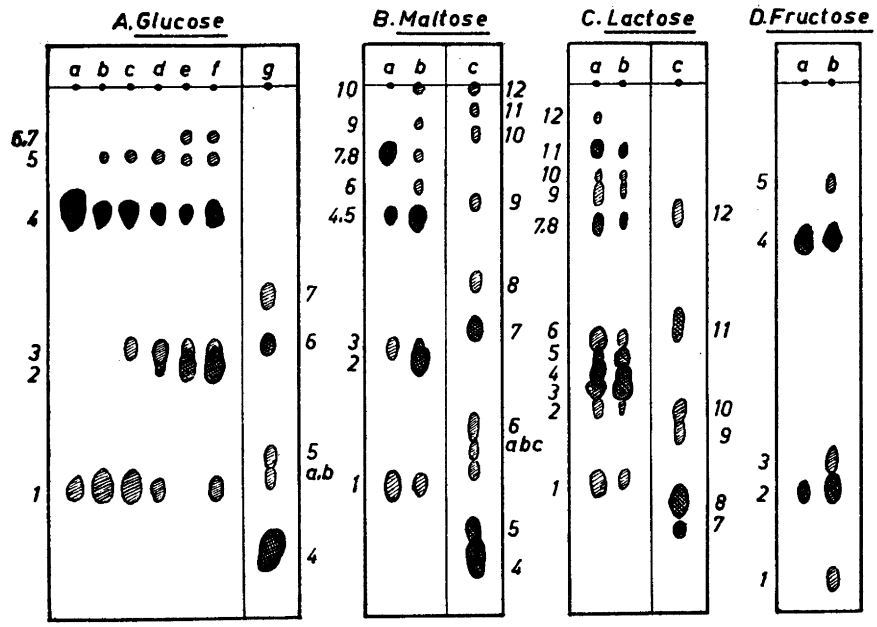

Fig. 2. Resin catalyzed methyl glycoside formation of mono- and disaccharides at 20° and 65° C.

A. Glucose. a. After 10 hours at 20° C. b. 15 h (20° C). c. 20 h (20° C). d. 300 h (20° C). e. 100 h (65° C). f. Mother liquor (65° C), chromatographed for 15 h. g. Same as in f, but chromatographed for 45 h.

1: Methyl glucofuranosides. 2: α-Methyl D-glucopyranoside. 3: β-Anomer of 2. 4: Glucose. 5 a, b: Disaccharide glycosides? 6: Trehalose (+ maltose?). 7: *Iso*maltose.

B. Maltose. a. 4 h (65° C). b. 50 h (65° C). c. Mother liquor (65° C).

1, 2, 3, 4: Same as for glucose. 5: ? 6 a, b, c: Disaccharide glycosides? 7: Trehalose + maltose. 8: *Iso*maltose. 9: Maltotriose? 10: Maltotetraose? 11: Maltopentaose? 12: ?

C. Lactose. a. 45 h (65° C). b. 95 h (65° C). c. Mother liquor (65° C).

1: Methyl glucofuranosides. 2: Methyl galactofuranosides. 3: α-Methyl glucopyranoside. 4: β-Anomer of 3. 5: α-Methyl galactopyranoside. 6: β-Anomer of 5. 7: Glucose. 8: Galactose. 9, 10: Disaccharide glycosides? 11: Lactose. 12: ?

D. Fructose. a. 1 h (65° C). b. 40 h (65° C).

1: Methyl fructofuranosides. 2: Methyl fructopyranosides. 3: ? 4: Fructose. 5: Difructose dianhydride?

the resin-catalyzed reaction being slower by a factor of about 10, at both temperatures. It might be reasonable to attribute this inferior activity of the ion exchanger, earlier demonstrated by Wadman[6], to the limited accessability for solute molecules to the active groups of the resin.

The minimum in the optical rotation curve obtained at room temperature in the mineral acid experiments[7] was found to coincide with the maximum percentage of furanoside isomers in the reaction mixture. Furanoside determinations proved this to hold true also in the present case, the maximum yield of furanosides being 50 % of total sugars.

The nature and the order of appearance of reaction products were revealed by paper chromatography (Fig. 2 A). Thus, methyl glucofuranosides were the first glucosides to appear, later followed by the β-glucopyranoside, which was then gradually converted into the α-anomer. No signs of other monosaccharide derivatives, as full acetals or septanosides, were observed. On the other hand, polysaccharide synthesis manifested itself already when only minor amounts of pyranosides had been formed. Distinct spots of *iso*maltose and trehalose were obtained, and it is probable that small amounts of maltose were present too, although obscured by the trehalose spot. In this case higher reversion products, containing more than two monosaccharide units, were not encountered.

α-Methyl D-glucopyranoside, constituting the main reaction product, was obtained in a practical yield of 60 % after percolating for 30 hours, the yield being increased to well above 80 % by recycling the mother liquor (*cf.* Fig. 1).

The behaviour of disaccharides towards the resin was tested on maltose and lactose. When percolated as glucose they gave reducing power *versus* time curves of the same shape as in Fig. 1 A, the rate of reaction, however, being lower. In the case of lactose the extremely low velocity was attributable to the slight solubility of the sugar in methanol.

According to paper chromatograms (Fig. 2 B and C) the gradual disappearance of maltose and lactose was not attended by a considerable disaccharide glycoside formation. Instead both sugars produced essentially methyl glycosides of the component monosaccharides, indicating the glycosidification of the disaccharides to be slow compared to the methanolysis reaction. The chromatograms of the maltose series closely resembled those of glucose but contained also some new spots in the low R_F region, evidently corresponding to reducing oligosaccharides with three and more monosaccharide units. Methyl gluco- and galactosides, in all their isomeric forms, comprised the main part of the lactose series chromatograms. In addition to lactose a few disaccharide spots were observed, but reversion products containing more than two monosaccharide units seemingly were absent in this case.

Fructose, being a representative ketose, was also subjected to methanol percolation. However, owing to its sensitivity to acids, the fructose molecule was rapidly degraded when exposed to the catalyst. The solution turned brown, and degradation products were deposited in the pores of the resin, thus causing a severe inactivation of the catalyst (Table 1).

Table 1. Resin catalyzed reaction between fructose and methanol at 65° C.

Reaction time hours	Reducing power per cent
0	100
1	8.0
3	4.5
5	4.4
22 *	4.0
42	3.7

* After 22 hours the used catalyst was discarded and replaced with fresh resin.

As appears from Table 1 substitution of the catalyst by fresh material only caused a temporary improvement. Some fructoside formation took place however (Fig. 2 D), but it was not possible to isolate any crystalline fraction from the reaction mixture.

*Iso*propylidene derivatives

Resin catalyzed condensations of glucose and fructose with acetone were carried out. It was found that fructose reacts rather rapidly at room temperature by merely being shaken with acetone and catalyst. An unduly prolonged reaction time is to be avoided because of the danger of degradation of fructose, this process being catalyzed by the acid decomposition products. Moreover, acetone itself may undergo condensation, and the materials formed are only difficultly separable from the sugar derivatives. — Already in about six hours most of the fructose had reacted, and in less than 24 hours a 40 % yield of 1,2-4,5-di*iso*propylidene fructofuranose was obtained, while only small amounts remained of the mono*iso*propylidene derivative first formed, as demonstrated paper-chromatographically. Only traces of other acetone fructose derivatives were observed.

The condensation of glucose with acetone requires a higher temperature to become of preparative value, and hence a modified technique involving soxhlet extraction with acetone of a glucose-resin-drierite mixture was employed. Since the acetone glucose derivatives are removed from the catalyst immediately after their formation, and since monoacetone glucose is formed more readily than the diacetonylated compounds the extraction technique enhances the relative proportion of the monoacetone derivative. The mixture resulting from a 24 hours extraction contained besides acetone-glucoses appreciable amounts of acetone autocondensation products. Resolution of the mixture by differential extraction yielded 1,2-mono- and 1,2-5,6-di*iso*propylidene glucofuranoses in roughly equal amounts.

The experiences gained with the ion exchange resins employed as catalysts indicate this technique to be a convenient method for the preparation of many sugar derivatives. Particularly methyl aldosides and acetone ketose derivatives may be advantageously produced in this way. Apparently the resin functions analogously to the strong acid in the Fischer type syntheses of glycosides and acetone derivatives.

Aldose isomerization

In the alkali induced isomerization of aldoses the equilibrium may be displaced towards the ketose by adding borate, as has been pointed out by Myrbäck [8], the operative effect being the high stability of the ketose-borate complexes involved.

This principle was applied to glucose, the isomerization being effected by alkaline borate buffers. When the transformation, as indicated by the optical rotation of the solution (Table 2), had come to an end, the mixture was examined paper-chromatographically (Fig. 3). Glucose and fructose in ample amounts — and their borate complexes — were present, but mannose, which ought to be formed according to the enediol mechanism of the Lobry de Bruyn transformation was not observed.

Most of the electrolytes were removed from the solution by ethanol precipitation, and after concentration of the filtrate the acetonylation technique was attempted to the isola-

Table 2. Isomerization of glucose at 20° C by means of alkaline borate. Composition of solution: glucose 0.56 M, *boric acid 0.63* M *and potassium hydroxide 0.88* M.

Time min	$[\alpha]_{20}^{D}$	Time min	$[\alpha]_{20}^{D}$
7	+ 20.4°	240	+ 15.0°
14	21.7	420	6.9
20	21.9	1 260	− 12.5
30	22.5	1 500	15.2
36	22.8	1 860	18.2
46	22.9	2 700	20.4
60	22.5	3 180	20.9
90	21.8	4 140	20.6
130	20.2		

tion of fructose. The process proved to be very slow, however, shaking for 95 hours with acetone and resin catalyst producing only minor amounts of *iso*propylidene fructose derivatives (Fig. 3). The acetonylation of pure fructose, on the other hand, proceeds rather rapidly, as shown above. Calcium hydroxide precipitation [9] following acid hydrolysis of the acetone derivatives was then tried but with negative result. Since the impeded reactivity of the fructose was regarded as a borate interference, the residual boric acid in the mixture was removed by complexing with catechol and subsequent anion exchange. As a matter of fact, when all boric acid was eliminated the fructose in the isomerization mixture was readily available to calcium hydroxide precipitation and to acetonylation (Fig. 3).

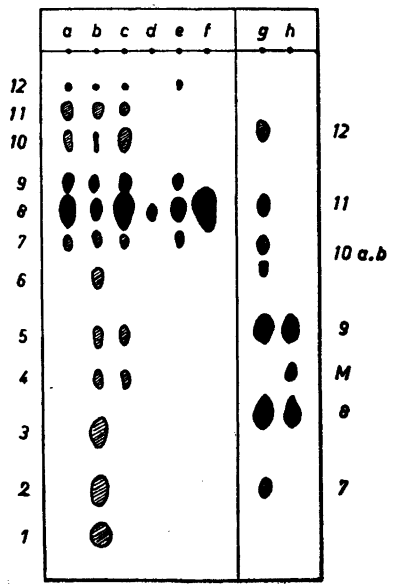

Fig. 3. Alkaline isomerization of glucose.

a. Isomerization mixture after 69 hours (chromatographed for 4 hours.) b. After acetonylation. c. After acid hydrolysis of acetonylation mixture. d. Fructose precipitated from c. with calcium hydroxide. e. Mother liquor from d. freed from borate. f. Fructose precipitated from e. with calcium hydroxide. g. Same as in a, but chromatographed for 20 hours. h. Fructose, mannose, and glucose.

1, 2, 3: *Iso*propylidene fructoses. 4, 5, 6: *Iso*propylidene glucoses. 7: ? 8: Fructose. 9: Glucose. 10 a, b: Fructose-borate complex. 11: Glucose-borate complex. 12: Difructose dianhydride? M: Mannose.

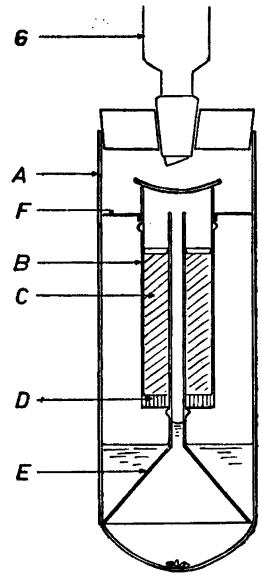

Fig. 4. Percolation apparatus. A. Boiling tube. B. Catalyst tube. C. Resin column. D. Sintered glass disc. E. Funnel supporting catalyst tube. F. Centering device for catalyst tube. G. Reflux condenser. Scale 1:4.

EXPERIMENTAL *

Materials. D-Glucose, anhydrous; maltose, monohydrate; lactose, monohydrate; D-fructose, all of *p. a.* quality. Methanol, absolute, purissimum, water content below 0.03 %. Acetone, purissimum, dried with drierite. Cation exchange resins, IR-120 and Dowex 50, in the hydrogen cycle.

Methods. For methyl glycoside synthesis the following standard procedure was employed:

In the boiling tube of the apparatus (borosilicate glass; Fig. 4) dissolve 25 g of the sugar in 400 ml of methanol by refluxing. Meanwhile prepare the catalyst column by placing a thin layer of glass wool in the bottom of the tube B and over that the resin (25 g) covered by another layer of glass wool. The stem of the supporting funnel E should protrude about an inch above the surface of the column. When the sugar is dissolved insert the prepared column, remount the condenser, and heat the boiling tube on a water bath. By boiling the solution is forced up through the stem of the funnel, then it percolates through the resin and finally drains back into the boiling tube through the sintered glass disc D. Bumping is avoided by placing broken quartz in the boiling tube. Percolation is continued, until the reducing power of the solution has attained a minimum value. The ensuing solution is crystallized fractionally. During the reaction aliquots are withdrawn for paper chromatographic examination and for the determination of reducing power and optical rotation.

The reducing power was determined by the Somogyi iodimetric micro method [10].

Paper chromatography was carried out on Whatman No. 54 paper, using ethyl acetate, acetic acid, water (60:17:17.5), and *n*-butanol, pyridine, water (3:2:1.5) mixtures as solvents. The silver reagent of Trevelyan *et al.*[11] was capable of detecting, in addition to reducing sugars, glycosides and other nonreducing materials, provided sufficient time was allowed for the reaction. By means of sodium sulfide black silver sulfide spots were eventually obtained. Alternatively, the naphthoresorcinol reagent [12] was used for the detection.

* All melting points were determined on the Kofler block.

Although not explicitly stated in every instance, running of parallell chromatograms with authentic specimens was used as a general method of characterization of reaction products.

Methyl glycosides

Glucose. Glucose (10 g) was shaken at 20° C with cation exchanger (10 g) and methanol (160 ml). Reducing power and optical rotation are given in Fig. 1 A and B, and paper chromatograms in Fig. 2 A.

At 65° C glucose (25 g) was reacted according to the standard procedure (Figs. 1 A and B and 2 A). In about 60 hours the reducing power had decreased to a constant value of about 0.5 % of the original value. In 30 hours 15 g of crude *α*-methyl glucoside were obtained, and recycling of the mother liquor for at least 30 hours yielded another 6 g of glucoside. Processing of the crystalline fractions and the mother liquors according to Patterson and Robertson [13] yielded in all 22 g (82 %) of *α*-methyl-D-glucopyranoside, m. p. 166° (from methanol), $[\alpha]_D^{20}$ +160° (c 0.5, in water), and 0.9 g of *β*-methyl glucoside, m. p. 103—7° (from methanol), $[\alpha]_D^{20}$ −32° (c 1.1, in water). The authenticity of the glucosides was confirmed by infra-red absorptiometry.

Maltose. Maltose (25 g) was percolated for 50 hours according to the standard procedure. Chromatograms are given in Fig. 2 B. In 50 hours the reducing power had decreased to 1.7 %. Working up of the solution yielded one crystalline fraction, m. p. 154—62° (from methanol), $[\alpha]_D^{20}$ +155° (c 0.5, in water), identified as *α*-methyl D-glucopyranoside (mixed melting point, paper chromatography, IR spectrum). Yield 14 g (48 %).

Lactose. By percolating a suspension of lactose (25 g) and methanol (400 ml) through the resin 15 g lactose could be brought into solution after 45 hours. After removal of undissolved lactose the solution was percolated further for 50 hours as above. Chromatograms are given in Fig. 2 C. The end value of the reducing power was 0.5 %. Upon crystallization *α*-methyl D-glucopyranoside, m. p. 161—5° (from methanol), and *α*-methyl D-galactopyranoside were obtained.

Fructose. The standard procedure was applied to fructose (25 g). The solution rapidly turned yellow, and then brown, and the catalyst became impregnated with black condensation products. The decrease of the reducing power finally stopped at about 4 % (Table 1), but the percolation was continued after substituting the resin by fresh material. After percolation the solution was decolourized by charcoal and concentrated to 25 ml. All attempts to crystallize the syrup failed, however. Chromatograms are given in Fig. 2 D.

*Iso*propylidene derivatives

Fructose. A mixture of fructose (8.0 g) and ion exchanger (8 g) was shaken at room temperature with acetone (150 ml), until the optical rotation of the solution became constant (24 hours). The solution was filtered, a little calcium carbonate was added, and the solvent was driven off at a reduced pressure. Extraction of the residue with an 1:20 ether-light petroleum (b. p. 40—60° C) mixture followed by crystallization yielded 4.4 g (38 %) of ("*α*")-1,2-4,5-di*iso*propylidene D-fructopyranose, m. p. 118° (from light petroleum), $[\alpha]_D^{20}$ −159° (c 0.5, in water). Trace amounts of the mono*iso*propylidene derivative were also present.

Glucose. A mixture of glucose (4.0 g), drierite (4 g) and resin catalyst (8 g) was extracted with boiling acetone (75 ml) for 25 hours in a soxhlet apparatus. After separation of unchanged glucose and acetone autocondensation products (2.5 g) the filtrate was evaporated to dryness at a diminished pressure. Most of the residual acetone condensation products were eliminated by treating the residue with water (50 ml) at room temperature and filtering through talc. The water was driven off from the filtrate at a reduced pressure and the residue on benzene extraction at room temperature and decolourization with charcoal yielded 1.6 g of 1,2-5,6-di*iso*propylidene D-glucofuranose, m. p. 105—7° (from *n*-heptan). The benzene-insoluble on room temperature extraction with acetone yielded 0.8 g of 1,2-monoisopropylidene glucose, m. p. 143—5° (from ethanol-light petroleum).

Aldose isomerization

10.0 g of glucose (56 millimoles) were dissolved in 25.0 ml of a borate buffer (2.50 *M* in boric acid and 3.50 *M* in potassium hydroxide) and the volume was adjusted to 100 ml with water. The solution was deaërated with nitrogen gas and was kept under nitrogen at 20° C. Part of the solution was filled in a polarimeter tube, and readings were taken, till the optical rotation became constant (70 hours, Table 2). Paper chromatographic examination of the solution then proved the presence of glucose and fructose as sole sugars. The pH of the faintly yellow solution was adjusted to 4 by means of sulfuric acid, and electrolytes were precipitated with 95 % ethanol (250 ml). The precipitate was washed on the filter with some ethanol and the filtrates were concentrated at a reduced pressure to a thin syrup whereby a great part of the residual boric acid was deposited. After filtration the syrup was concentrated *in vacuo* to dryness, the water being driven off as the ternary azeotrope with ethanol and benzene.

The dried and powdered reaction product (8.0 g) was shaken at room temperature with ion exchanger (10 g) and acetone (150 ml) for 95 hours. After 40 hours drierite (5 g) was added. The reaction mixture contained, in addition to glucose and fructose and their borate complexes, small amounts of fructose *iso*propylidene derivatives (Fig. 3). The solution was filtered from the catalyst, a little calcium carbonate was added, and the solvent was driven off at a reduced pressure. The residue was leached with water (100 ml) at room temperature, the mixture was filtered through charcoal, and the dissolved *iso*-propylidene derivatives were hydrolyzed by 0.01 *N* hydrochloric acid at 85° for 18 hours. The hydrolysate was precipitated at +5° C with powdered calcium hydroxide (6 g), and the precipitate was washed on the filter with water (3 × 10 ml) and then suspended in 100 ml of water and decomposed with 2.5 *M* sulfuric acid (22 ml). After removal of the calcium sulfate, the pH of the solution was adjusted to 4, and the water was evaporated at a reduced pressure. The residue (0.5 g) contained only traces of fructose (Fig. 3).

The filtrate from the above calcium hydroxide precipitation gave a distinct borate reaction. Catechol (6 g) and potassium carbonate (2 g) were added, and the solution was filtered and passed through a column of anion exchanger (IR-4B, in the hydroxyl cycle). The eluate was acidified with hydrochloric acid, and excess catechol was removed by ether extraction.

Two thirds of the borate-free eluate were subjected to the calcium hydroxide precipitation, and decomposition of the precipitate yielded 2.5 g of fructose. The remainder of the eluate was brought to pH 4 and, after evaporation to dryness at a reduced pressure, was acetonylated as above. Di*iso*propylidene fructose was readily formed.

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The Kinetics and Mechanism of the Reaction between Cerium(III) and Persulphate

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The kinetics of the thermal decomposition of persulphate in aqueous solutions and of the redox reaction between cerium(III) and persulphate are investigated in a perchlorate ionic medium and in a mixed perchlorate-sulphate medium. It is found that the oxidizing agent in the redox reaction is the intermediate radical ion SO_4^-, formed in a decomposition reaction of persulphate that is uncatalyzed by hydrogen ions. Evidence is also given that only one SO_4^- can be formed from every persulphate ion decomposing in this way.

The mechanism previously proposed for the persulphate decomposition is changed so as to be consistent with these results.

The fact observed that cerous sulphate complexes are oxidized more rapidly than the hydrated cerous ion is discussed in terms of an electron transfer mechanism.

The thermal decomposition of persulphate in aqueous solutions has been the subject of several investigations (*cf.* Kolthoff and Miller [1]). The nature of some of the intermediate products formed in the decomposition has been convincingly established, but the mechanism proposed for the reaction has not been tested in more detail.

In preliminary measurements the present authors found that the oxidation of cerous salts by persulphate in the absence of catalyzing silver ions is noticeable only at such temperatures where the thermal decomposition of persulphate is significant. For this reason the present investigation was started with the aim of elucidating the mechanism of this redox reaction and in this way also of giving further information about the mechanism for the thermal decomposition of persulphate. Furthermore, a study of this oxidation of cerium(III) is the natural introduction to a close investigation of the corresponding reaction in the presence of silver ions.

EXPERIMENTAL

Chemicals. From calculated amounts of ammonium persulphate, Merck's p. a., solutions were prepared just before use.

Cerous perchlorate was obtained by heating cerous chloride with 70 % perchloric acid. The preparation obtained was free from chloride ions and was recrystallized from water.

A stock solution of ceric perchlorate was prepared by anodic oxidation at platinum of a cerous perchlorate solution, acidified with perchloric acid. After the oxidation the ceric concentration was about 75 % of the total cerium concentration.

Perchloric acid, sodium perchlorate, and sodium sulphate were of analytical grade.

The redox reaction. Two solutions, one containing cerous perchlorate and perchloric acid and the other containing ammonium persulphate, sodium sulphate (in certain experiments) and sodium perchlorate, were kept for thirty minutes in a paraffin-oil thermostat at 61° C. Then equal volumes were mixed, and after certain time intervals samples of 5 ml were withdrawn by pipette and added to an equal volume of ice-cooled water. After tempering in a thermostat at 20° C for thirty minutes the light extinction of the solution was determined at the wave length 400 mμ with a Hilger Uvispek Spectrophotometer or a Beckman Quartz Spectrophotometer (Model DU). The layer thickness was always 1 cm.

To determine the values of the molar extinction of cerium(IV) in the different samples of a measurement series it was necessary to perform a calibration series with the cerium (III) exchanged for the ceric-cerous mixture with 75 % cerium(IV) but otherwise with the same ionic composition. In a third measurement series of the same kind the cerium was initially present only as cerium(III) and in the same concentration as in the calibration series. The difference between the extinction values of the two last-mentioned series at a given time yields the extinction corresponding to the initial ceric concentration in the calibration series. Then the ceric concentrations in the different samples were obtained from their extinctions.

The persulphate decomposition. At the acid concentrations used the thermal decomposition of persulphate in aqueous solutions with the liberation of oxygen is rather rapid at 61° C, and for this reason a separate investigation of the kinetics of this decomposition was performed.

The solutions were prepared as in the measurements described above, and samples of 5 ml were withdrawn and added to the same volume of ice-cooled water in order to practically stop the reaction. From 5 ml of this mixture oxygen was removed by bubbling for ten minutes with purified nitrogen. Then 5 ml of dilute sulphuric acid, 5 ml of 0.1 *M* ferrous sulphate and about 50 ml of water of 70 – 80° C were added, causing a rapid reduction of the persulphate. An excess of ceric sulphate, strongly acidified with sulphuric acid, was added, and after cooling to room temperature the excess of ceric sulphate was titrated with ferrous sulphate, ferroin being used as indicator.

When the samples withdrawn contained cerium, the method described gave the sum of the persulphate and ceric concentrations. Then the ceric concentration, previously determined, was subtracted.

THE RATE LAWS FROM THE MEASUREMENTS

The following symbols for concentrations are used.

a = the initial concentration of cerium(III).
$b(t)$ = the concentration of persulphate at the time t.
x = the concentration of cerium(IV).
y = the concentration of an intermediate species, denoted A.

Kinetics of the persulphate decomposition. The rate of decomposition of persulphate was studied for different values of $b(0)$, a and the hydrogen ion concentration. In Fig. 1 ln $b(t)$ is plotted against t. Straight lines are obtained, indicating a first order reaction in every measurement series. For the ionic medium 500 m*M* $HClO_4$ + 500 m*M* $NaClO_4$, the rate constant $k_0 = (5.0 \pm 0.1) \times 10^{-3}$ min^{-1} is obtained. When the ionic medium has the composition 500 m*M* $HClO_4$ + 150 m*M* Na_2SO_4 + 50 m*M* $NaClO_4$, we get $k_0 = (3.8 \pm 0.1) \times 10^{-3}$

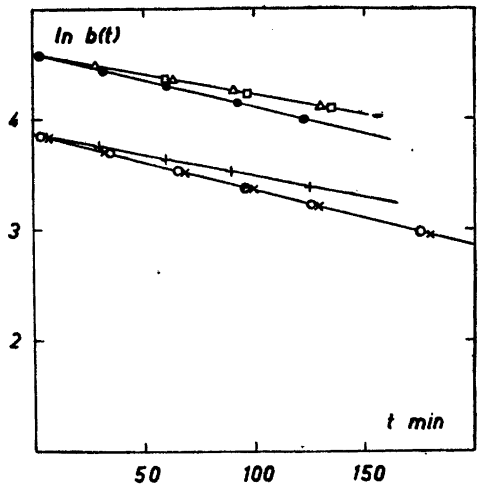

Fig. 1. ln b(t) as a function of t at different values of b(0) (48.2 and 98.6 mM), a, and $[H^+]$. *a = 0,* $[H^+]$ *= 500 mM (● and ○); a = 1.74 mM,* $[H^+]$ *= 500 mM (×); a = 0,* $[H^+]$ *= 378 mM (△ and +); a = 1.74 mM,* $[H^+]$ *= 378 mM (□).*

min^{-1}. In the last-mentioned case the hydrogen ion concentration, determined by measurements with the quinhydrone electrode, had the value 378 m*M*.

Since $HS_2O_8^-$ is a strong acid (*cf.* Kolthoff and Miller [1]) a rather small addition of persulphate to the ionic medium cannot change the $[H^+]$-values appreciably. Thus, within the limits of the experimental random errors the k_0-values obtained are proportional to the $[H^+]$-values and independent of the small amounts of cerium in the solutions.

Kolthoff and Miller [1] have found that in alkaline and neutral solutions the decomposition is slow with the value 3.0×10^{-4} min^{-1} for the rate constant at 60° C (corresponding to 3.5×10^{-4} min^{-1} at 61° C, calculated from the activation energy given [1]), independent of the values of pH and the ionic strength. Thus persulphate decomposes by two competing reactions, one uncatalyzed and the other catalyzed by hydrogen ions, and then in our measurements the correct expression for k_0 is:

$$k_0 = 3.5 \times 10^{-4} + 1.00 \times 10^{-2} \, [H^+] \text{ min}^{-1} \qquad (1)$$

Kinetics of the redox reaction. In Fig. 2 the concentration x of cerium(IV) has been plotted against t with a and $b(0)$ as parameters and with perchloric acid and sodium perchlorate in constant concentrations as additional electrolytes. From the dependence of dx/dt at $t = 0$ upon a and $b(0)$ it is easily found that the formation of cerium(IV) is a first order reaction with respect to persulphate but of no simple order with respect to cerium(III), since the dependence on a decreases with increasing a-values. Thus it is natural to try to represent the rate expression by a differential equation of the form:

$$\frac{dx}{dt} = \frac{k \, (a-x) \, b \, (t)}{1 + a \, (a-x)} \qquad (2)$$

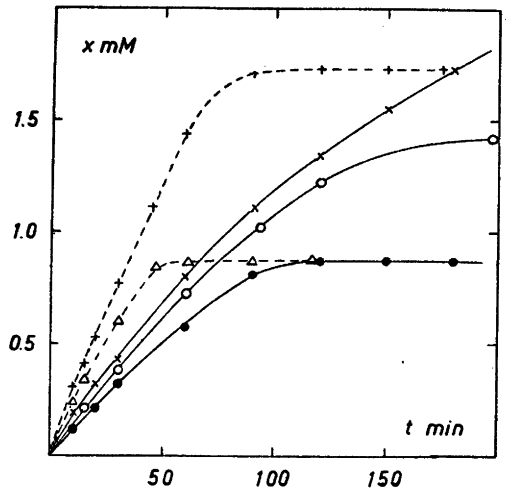

Fig. 2. The relation between x and t at different values of a and b(0) and with 500 mM $HClO_4$ + 500 mM $NaClO_4$ as additional electrolytes. a = 0.870 mM, b(0) = 48.2 mM (●); a = 1.74 mM, b(0) = 48.2 mM (○); a = 3.47 mM, b(0) = 48.2 mM (×); a = 0.870 mM, b(0) = 98.6 mM (△); a = 1.74 mM, b(0) = 98.6 mM (+).

where k and α are constants. If we make use of the expression for $b(t)$, an integration of eq. (2) yields:

$$\frac{k_0}{b(0)-b(t)} \ln \frac{a}{a-x} = k - \frac{\alpha k_0 x}{b(0)-b(t)} \tag{3}$$

For the sake of brevity we put $u = k_0x/(b(0)-b(t))$ and the left member of eq. (3) equal to z. Then, if the rate expression proposed is correct, all the measurement series should give one and the same linear relation between u and z.

In Table 1 corresponding values of u and z have been calculated for such t-values that the differences $b(0)-b(t)$ and $a-x$ are not influenced very greatly by the experimental random errors. In Fig. 3 the function z has been plotted against u, and it is seen that a straight line is obtained. Thus, for the concentration ranges used the rate law, represented by eq. (2), is verified. The intercept on the u-axis is well defined and yields $k/\alpha = (3.4 \pm 0.1) \times 10^{-4}$ min^{-1}, and from the slope of the line we get $\alpha = (3.5 \pm 0.2) \times 10^3$ M^{-1}.

In Fig. 4 the formation of cerium(IV) is represented when the additional electrolytes are perchloric acid, sodium sulphate, and sodium perchlorate. It is evident that for $a-x > 0.2$ mM x is independent of the a-value. This means that the term $\alpha(a-x)$ in eq. (2) is $\gg 1$ in this case, and k/α can be obtained from the slope of the approximately straight-lined, first part of the curves according to the equation:

$$\lim_{t \to 0} \frac{dx}{dt} = \frac{k\,b(0)}{\alpha} \tag{4}$$

Table 1. Calculated values of u and z in the different measurement series at selected values of t. Additional electrolytes:

500 mM $HClO_4$ + 500 mM $NaClO_4$.

a mM →	0.870			1.74			3.47		
$b(0)$ mM →	48.2			48.2			48.2		
t min	x	$u \cdot 10^3$	z	x	$u \cdot 10^3$	z	x	$u \cdot 10^3$	z
30.0	0.32	0.240	0.345	0.38	0.285	0.185	0.42	0.315	0.095
45.0	0.45	0.230	0.370						
60.0	0.57	0.225	0.420	0.71	0.280	0.205	0.80	0.315	0.100
90.0				0.99	0.280	0.235	1.10	0.310	0.105
120.0							1.34	0.305	0.110

a mM →	0.870			1.74		
$b(0)$ mM →	98.6			98.6		
t min	x	$u \cdot 10^3$	z	x	$u \cdot 10^3$	z
15.0	0.34	0.235	0.345	0.41	0.285	0.190
30.0	0.60	0.220	0.430	0.77	0.280	0.215
45.0				1.11	0.270	0.250

x expressed in mM

u » » min^{-1}

z » » $min^{-1} M^{-1}$

The measurements give $k/a = (3.7 \pm 0.1) \times 10^{-4}$ min^{-1}. When the concentration of sodium perchlorate was increased to compensate for the decrease in ionic strength caused by the formation of hydrogen sulphate ions, we obtained $k/a = (3.3 \pm 0.1) \times 10^{-4}$ min^{-1}.

For $t = 50$ min, $a = 0.87$ mM and $b(0) = 48.2$ mM we can compute $z = 1.4$ min^{-1} M^{-1}, while the u -value is equal to k/a within the limits of the random errors. Thus, if these are taken into consideration we have $k/a - u \leq 0.2 \times 10^{-4}$ min^{-1}, and then eq. (3) yields $a \geq 7 \times 10^4$ M^{-1}.

It is evident that a partial exchange of perchlorate ions for sulphate ions at a constant ionic strength has no influence upon k/a, whereas the a-value increases considerably.

THE MECHANISM OF THE OXIDATION-REDUCTION REACTION

From the rate law verified it is seen at once that the reaction between cerium(III) and persulphate is not a simple bimolecular one. Neither can it be such a reaction preceded by a formation of an ordinary complex between these components, for in this case the rate law would have been of the form

$$\frac{dx}{dt} = \text{constant} \cdot \frac{(a - x)\, b\,(t)}{1 + \beta\, b\,(t)} \qquad (5)$$

if β is the complexity constant, and provided persulphate is in great excess.

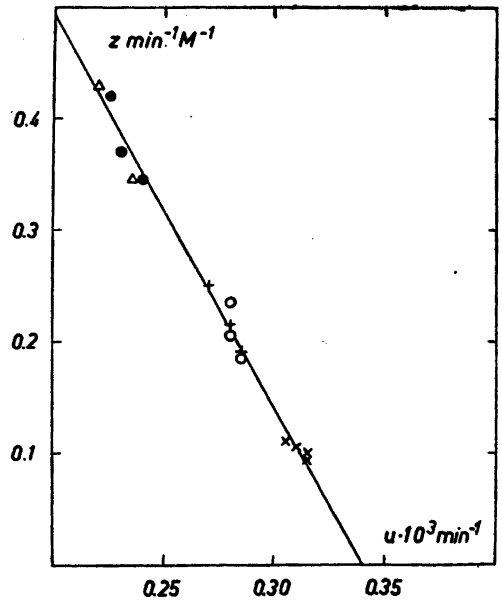

Fig. 3. z as a function of u. The signs used relate to the same concentrations as in Fig. 2.

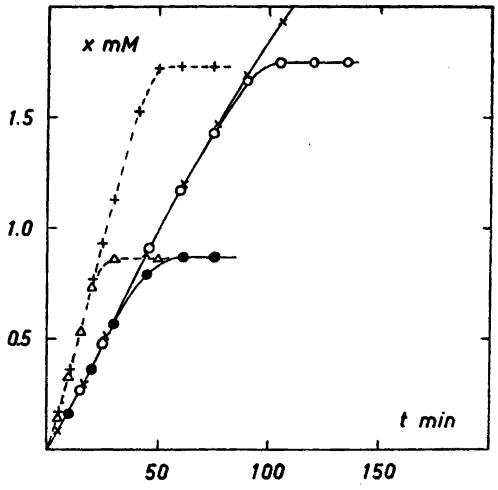

Fig. 4. The relation between x and t at different values of a and b(0) and with 500 mM $HClO_4$ + 150 mM Na_2SO_4 + 50 mM $NaClO_4$ as additional electrolytes. a = 0.870 mM, b(0) = 48.2 mM (●); a = 1.74 mM, b(0) = 48.2 mM (○); a = 3.47 mM, b(0) =, 48.2 mM (×); a = 0.870 mM, b(0) = 98.6 mM (△); a = 1.74 mM, b(0) = 98.6 mM (+).

Then the only possible explanation of the reaction is that the oxidizing agent is an intermediate species A, formed in the thermal decomposition of persulphate. The rate constant for the formation of A is denoted by k_1, and depending on whether the ions (or molecules) A are formed in the uncatalyzed decomposition reaction or in the one catalyzed by hydrogen ions, the value of k_1 must be equal to either $3.5 \times 10^{-4} \times n$ min^{-1} or $1.00 \times 10^{-2} \times [H^+] \times n$ (that is approximately $k_0 \times n$) min^{-1}, where n is an integer, indicating how many A are formed from one persulphate ion.

In the absence of cerium(III) A reacts under the liberation of oxygen, and for this partial process we introduce the rate constant k_2. In the presence of cerous ions and cerous sulphate complexes (*cf.* Fronæus [2]) we have a set of simultaneous redox reactions of the type

$$Ce(SO_4)_j^{3-2j} + A \rightarrow Ce(SO_4)_j^{4-2j} + A^- \qquad (j = 0, 1, \cdots) \tag{6}$$

Then for the rates of formation of A and cerium(IV) the following equations can be expected to be applicable.

$$\frac{dy}{dt} = k_1\ b(t) - k_2\ y - k_3\ (a-x)\ y \tag{7}$$

$$\frac{dx}{dt} = k_3\ (a-x)\ y \tag{8}$$

Here the coefficient k_3 is a function of the sulphate ion concentration, if the cerous ion and the cerous sulphate complexes have different individual rate constants for the redox process (6).

If $k_3(a-x)$ is a quantity that is negligible in comparison with k_2 the system of differential equations (7, 8) has a simple solution. Then, if $y = y_0$ when $t = 0$ (the time at which cerium(III) is added), and we apply the relation $b(t) = b(0)\ e^{-k_0 t}$ verified before, the simplified equation

$$\frac{dy}{dt} + k_2\ y = k_1\ b\,(t) \tag{9}$$

has the solution

$$y = \left(y_0 - \frac{k_1\ b\,(0)}{k_2 - k_0} \right) e^{-k_2 t} + \frac{k_1\ b\,(0)}{k_2 - k_0} e^{-k_0 t} \tag{10}$$

If the condition $k_2 \gg k_0$ is fulfilled the last term in the right member of eq. (10) will be the dominating one a short time after the starting of the redox reaction and we have approximately: $k_2 y = k_1 b(t)$. Comparing this expression with eq. (9) we find that for such t-values dy/dt is a quantity negligible in comparison with $k_2 y$. This is the steady-state approximation.

When $k_3\ (a-x)$ cannot be neglected, $k_2 + k_3\ (a-x)$ varies with time, but provided this sum is $\gg k_0$, it is obvious that the steady-state approximation must still be applicable, giving us: $y\{k_2 + k_3\ (a-x)\} = k_1 b(t)$. Combining this expression with eq. (8) we obtain

$$\frac{dx}{dt} = \frac{k_1\ k_3\ (a-x)\ b(t)}{k_2 + k_3\ (a-x)} \tag{11}$$

With $k_1 = k/\alpha$ and $k_3/k_2 = \alpha$ this differential equation is identical with the rate law (2), experimentally verified.

Thus k_1 has the value 3.4×10^{-4} min^{-1}, independent of the variation in $[H^+]$ accompanying the partial exchange of perchlorate for sulphate ions. Since this value within the limits of experimental random errors is equal to the rate constant of the uncatalyzed thermal decomposition of persulphate but much smaller than k_0, we can conclude that the species A is formed in this reaction, and that *only one* A is formed from every persulphate ion decomposing in this way.

Furthermore, the great increase in k_3/k_2 on the addition of sulphate ions must certainly be due to an increase in k_3, since this change in the ionic medium of a constant ionic strength cannot possibly cause a very great decrease in the activity coefficients included in k_2. This means that the cerous sulphate complexes have greater values for the rate constant of the redox process (6) than the free hydrated cerous ion.

DISCUSSION

For the uncatalyzed thermal decomposition of persulphate in aqueous solutions Bartlett and Cotman [3] have proposed the following rate-determining step

$$S_2O_8^{2-} \rightarrow 2\ SO_4^- \qquad (12)$$

followed by a reaction between the radical ion SO_4^- and water, giving the intermediate radical OH and finally oxygen. This mechanism, involving a symmetrical dissociation of the persulphate ion, has been accepted by Kolthoff and Miller [1]. There is also much evidence in other investigations [4,5] that SO_4^- is the strongly oxidizing agent formed in the uncatalyzed thermal decomposition of persulphate ions, but no urgent reasons for the acceptance of all steps in this mechanism exist.

It is evident that our intermediate species A is equal to the radical ion SO_4^-, and then another mechanism, consistent with our conclusion that only one SO_4^- can be formed from one persulphate ion, must be introduced. For the uncatalyzed thermal decomposition and the redox reaction with cerium(III) we propose the following mechanism.

$$S_2O_8^{2-} + H_2O \xrightarrow{k_1} HSO_4^- + SO_4^- + OH \qquad (13)$$

$$SO_4^- + H_2O \xrightarrow{k_2} HSO_4^- + OH \qquad (14)$$

$$(k_2 \gg k_1)$$

$$Ce(SO_4)_j^{3-2j} + SO_4^- \xrightarrow{k_3} Ce(SO_4)_j^{4-2j} + SO_4^{2-} \qquad (15)$$

$$(j = 0,\ 1, \cdots)$$

$$2\ OH \rightarrow H_2O + \tfrac{1}{2}\ O_2 \qquad (16)$$

Bartlett and Nozaki [5] have shown that in alkaline solutions the rate constant of the decomposition of persulphate increases considerably (more than eight-fold at 80° C), if the solution is saturated with ethyl acetate. This makes it very plausible that the rate-determining step is a bimolecular reaction involving the solvent, as we have assumed in (13).

Since our kinetics data do not indicate any reduction of the cerium(IV) formed in the step (15), we must conclude, if the mechanism proposed is correct, partly that the radical OH cannot reduce cerium(IV) in these solutions, partly that OH does not form hydrogen peroxide. The first of these conclusions is consistent with results obtained by Milling, Stein, and Weiss (*cf.* Weiss [6]), and the second conclusion is supported by an investigation of Milling and Weiss (*cf.* Weiss [6,7]). The reaction (16) must not be interpreted as a simple step, it only indicates that the OH radical yields free oxygen.

The mechanism is also in agreement with the results of Kolthoff and Miller [1], *e. g.* that the oxygen liberated by the uncatalyzed decomposition of persulphate comes from the water and not from the persulphate.

When it is a question of explaining the result obtained that cerous sulphate complexes are oxidized more rapidly than the free hydrated cerous ion, several factors should be taken into consideration.

It is seen at once that the Coulomb forces between the reactants in step (15) must be of little significance for the reaction rate, since this attraction decreases or is changed into repulsion by the coordination of sulphate ions.

On the other hand, the application of the Franck-Condon principle [8] to the redox step (15) can give a conceivable explanation. Since the cerous and ceric ions have different energies of hydration, the rearrangement of the hydration atmosphere connected with the electron transfer involves an energy barrier to the reaction. By the coordination of sulphate ions the energies of hydration and the barrier certainly are decreased, and thus the reaction rate can be increased.

It is also conceivable that by the coordination of sulphate ions the 4f electron is promoted to a 5d electron, which should increase the probability for electron transfer.

Finally we will discuss the ability of the ligands to act as electron conductors. If the reaction step (14) occurs with a water molecule coordinated to a cerous ion the reaction does not proceed to oxidation of the central ion, since according to our mechanism above OH does not react with cerium(III). On the other hand, when an electron is transferred from a coordinated sulphate ion to a radical ion SO_4^- the oxidizing agent comes close to the central ion, and the redox reaction (15) can occur. Thus we must conclude that in the present case the sulphate ligand is a better electron conductor than the water ligand, and an increased rate of the redox reaction by the coordination of sulphate ions is to be expected.

This investigation has been supported by a grant from the *Swedish Natural Science Research Council* which is gratefully acknowledged.

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X-Ray Investigations of Reductone, Reductonates and Bromomalonic dialdehyde

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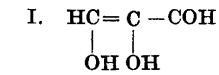
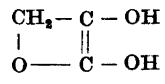
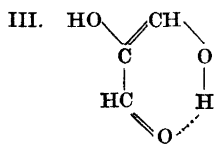
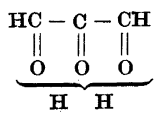
X-ray investigations have been made on reductone and on a series of related compounds. Only the main results are given in this paper. The crystal structure for bromomalonic dialdehyde, $C_3H_3O_2Br$, has been solved and a Fourier projection ϱ (xyp) has been calculated for rubidium reductonate, $C_3H_3O_3Rb \cdot H_2O$. Both compounds showed the same general form of the organic molecule. Therefore we expect a similar arrangement for reductone itself and are now attacking the problem of solving its structure from this point of view.

In his book "Reduktone" von Euler [1] has given four possible structure formulae for reductone, $C_3H_4O_3$, namely:

I. 

II. 

III. 

IV. 

where formula IV implies that all oxygen atoms in the molecule are equivalent by resonance. Formula III was originally proposed by Arndt [3]. All these formulae were considered as equally possible from a chemical point of view. A determination of the crystal structure of reductone might, however, show which of them is correct. At Professor von Euler's request we therefore started a crystallographic investigation of reductone.

Single crystals of reductone (kindly supplied by Professor von Euler and Mr. Hasselquist) were investigated by rotation and Weissenberg photographs (*hk*0, *hk*1, *h*0*l*, *h*1*l*, 0*kl*, and 1*kl*) using Cu-*K* radiation. They were found to be of orthorhombic symmetry with the following unit cell dimensions:

$$a = (10.5 \pm 0.1)\ \text{Å}$$
$$b = (10.0 \pm 0.1)\ \text{Å}$$
$$c = (3.6 \pm 0.1)\ \text{Å}$$
$$V = 380\ \text{Å}^3$$

In the photographs the following reflections were systematically absent:

$hk0$ with h = odd
$0kl$ with $k + l$ = odd

which is characteristic of the space groups No. 33 $Pn2_1a$ [4] and No. 62 $Pnma$ [4]. The density, as given in the monograph "Reduktone" [2] is 1.38, indicating that there are 3.59 (~ 4) formula units per unit cell.

From Patterson projections ($P(upw)$, $P(uvp)$ and $P(pvw)$) and "trial and error" calculations we found that the intensities of the reflections could not be explained assuming $Pnma$. The correct space group should then be $Pn2_1a$ which is non-centrosymmetric. The determination of the structure would therefore be rather difficult, as there was no previous knowledge about the spatial arrangement of reductone.

We therefore tried some related compounds, *viz.* reductonates and bromomalonic dialdehyde (kindly supplied by Professor von Euler and Mr. Hasselquist), in the hope of finding crystals which have more favourable symmetry. Table 1 summarizes the crystallographic data obtained from rotation and Weissenberg photographs of these compounds.

Of these compounds there are only two, $C_3H_3O_3Na$ and $C_3H_3O_3Rb \cdot H_2O$, that unambiguously have centres of symmetry. However, it was thought to

Table 1.

Compound	Crystal system	Unit cell dimensions	Characteristic space groups	Formula units per unit cell
Reductone ($C_3H_4O_3$)	Orthorhombic	$a = 10.5$ Å $b = 10.0$ Å $c = 3.6$ Å $V = 380$ Å^3	No. 62 $Pnma$ No. 33 $Pn2_1a$ **	4
Sodium reductonate ($C_3H_3O_3Na$)	Monoclinic	$a = 9.8$ Å $b = 11.6$ Å $c = 3.5$ Å $\beta = 96°$ $V = 397$ Å^3	No. 14 $P2_1/n$ **	4 *
Potassium reductonate ($C_3H_3O_3K$)	Triclinic	$a = 10.0$ Å $b = 11.3$ Å $c = 3.9$ Å $\alpha = 92°$ $\beta = 98°$ $\gamma = 96°$ $V = 440$ Å^3	No. 2 $P\bar{1}$ No. 1 $P1$	4 *
Rubidium reductonate ($C_3H_3O_3Rb \cdot H_2O$)	Monoclinic	$a = 8.3$ Å $b = 18.3$ Å $c = 4.0$ Å $\beta = 93°$ $V = 607$ Å^3	No. 14 $P2_1/a$	4 *
Bromomalonic dialdehyde ($C_3H_3O_3Br$)	Orthorhombic	$a = 6.4$ Å $b = 10.7$ Å $c = 6.3$ Å $V = 431$ Å^3	No. 63 $Cmcm$ No. 40 $C2cm$ ** No. 36 $Cmc2_1$	4 *

* By comparison with reductone.
** Orientation, different from that given in the International Tables.

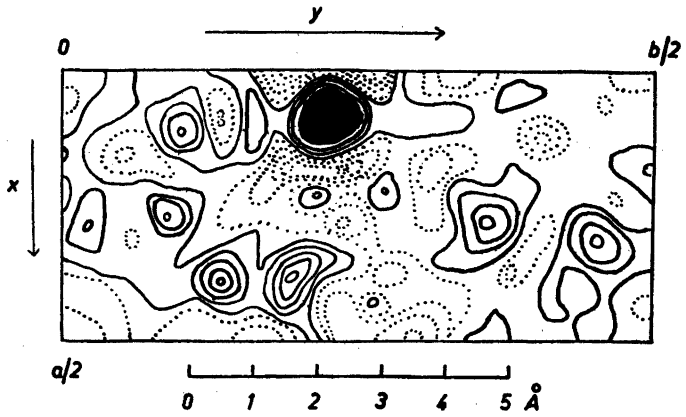


Fig. 1. Electron density projection on (001) of the crystal structure of $C_3H_3O_3Rb \cdot H_2O$.

be easier to find the structure of the rubidium salt, and this was therefore chosen as a suitable subject for the investigation. A full report of the calculations will be published later on; in this paper we are only giving the results obtained.

Fig. 1 shows the final electron density projection $\varrho\,(xyp)$ of $C_3H_3O_3Rb \cdot H_2O$. Only this projection was calculated, as the c axis is very short and the general shape of the molecule should appear rather well resolved there. Fig. 2 shows a diagram of the structure, projected on the xy plane. It is seen that the alternative structures I and IV for reductone are the only ones compatible with the projection, if it is assumed that the reductonate ion has the same configuration as reductone itself. It is also seen that, in the molecules, all three oxygen atoms are situated on the same side of the C—C chain. However, we could not decide between I and IV, as the z parameters of the atoms were not determined

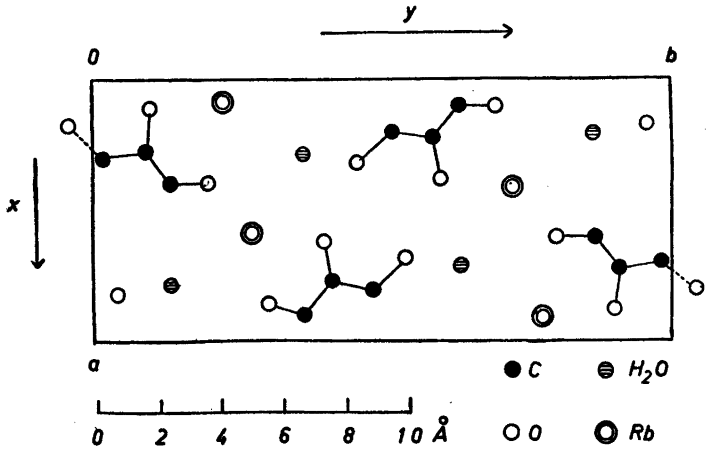


Fig. 2. Diagram of the crystal structure of $C_3H_3O_3Rb \cdot H_2O$ *projected on (001).*

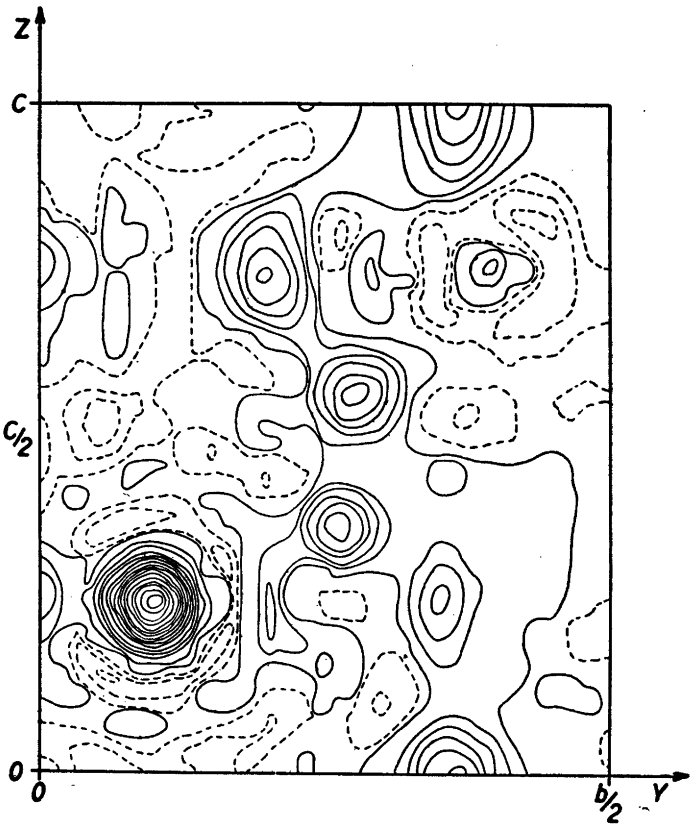


Fig. 3. Electron density in the plane $x = 0$ for bromomalonic dialdehyde.

and consequently the distances and bond angles within the reductonate ion could only be roughly estimated.

To see if this general appearance of the C_3O_3 skeleton was restricted to rubidium reductonate or if it could also be found in other reductone compounds, we investigated the crystal structure of a reductone derivative in which one of the OH groups had been substituted by a bromine atom. Its unit cell, Laue symmetry and characteristic space groups were obtained from single crystal photographs with Cu-K radiation and are given in Table 1. The correct space group was found to be $Cmc2_1$, which is non-centrosymmetric and thus rather unfavourable from our point of view. However, we could show that the C_3O_2Br skeleton in this compound is planar and that it is situated in a plane of symmetry. It was then possible to determine its structure.

Fig. 3 shows the electron density ϱ $(0yz)$ in the plane $x = 0$, which is the plane of the molecule. A diagram of the molecular arrangement is shown in Fig. 4. It is seen that the general arrangement of the C_3O_2Br skeleton is the same as in rubidium reductonate, *i. e.* 2 O + 1 Br are situated on the same side of the C—C chain.

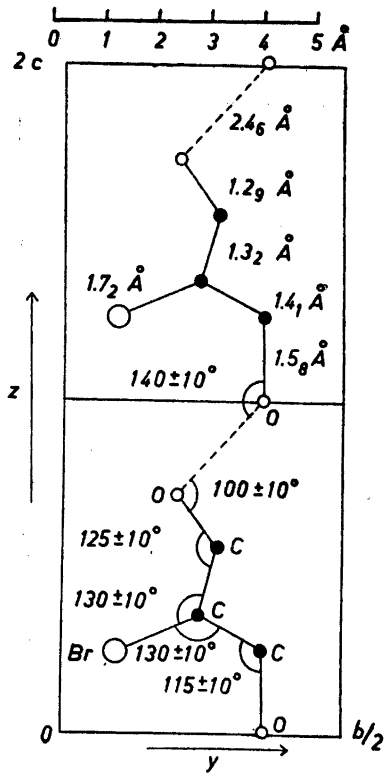


Fig. 4. Diagram of the crystal structure of bromomalonic dialdehyde, giving the bond angles and interatomic distances within a molecule.

The distances and bond angles within a molecule are also given in Fig. 4. Owing to the large scattering power of the bromine atom the accuracy in the determination of the carbon and oxygen parameters is too low to enable us to make a decision between the alternative structures I and IV for reductone. A detailed description of the structure of bromomalonic dialdehyde will appear elsewhere.

As both $C_3H_3O_3Rb \cdot H_2O$ and $C_3H_3O_2Br$ show the same general shape of the organic molecule, we might expect that a similar arrangement would also be found in reductone itself, and we are now attacking the problem of solving its crystal structure from this point of view.

We are much indebted to Professor Hans von Euler for his suggesting the problem and for his kind continuous interest. We also wish to thank Professor Lars Gunnar Sillén for valuable discussions.

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Studies on the Chemistry of Lichens

VIII *. Investigation of a *Dermatocarpon* and Some *Roccella* Species

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The systematic investigation of low-molecular carbohydrates in lichens [1] has been extended to a *Dermatocarpon* and some *Roccella* species. From *D. miniatum* mannitol, volemitol, α,α-trehalose and sucrose were isolated. From the *Roccella* species erythritol, arabitol, mannitol, galactose and the internal salt choline sulphate, were isolated. The presence of the rare sugar tagatose, combined with galactose in the form of a di- and a tri-saccharide was indicated.

In Part IV [1] of this series the investigation of the low-molecular carbohydrate constituents of a number of lichens, using chromatographic methods, was reported. Most lichens were investigated by paper chromatography only, but for some of them, all belonging to the order *Gymnocarpeae*, the compounds were separated on carbon columns and isolated as crystalline substances. The genus *Roccella* of this order, which is known to contain erythritol and thus differs from the others, was not represented. In the present paper investigations of *Dermatocarpon miniatum*, order *Pyrenocarpeae*, and three *Roccella* species are reported.

From *D. miniatum* D-mannitol (0.4 %), D-volemitol (4 %), α,α-trehalose (1.1 %) and sucrose (0.17 %) were isolated. Mannitol and volemitol were detected in the previous investigation of *Dermatocarpon* species, but only the volemitol was isolated and the present isolation of mannitol is complementary to these results. The disaccharides are the same as those found in lichens of the order *Gymnocarpeae*.

As mentioned above, lichens of the genus *Roccella* are known to contain erythritol and thus differ from other lichens of the order *Gymnocarpeae*. In the previous investigation D-arabitol and D-mannitol were found in all lichens belonging to this order. Now three *Roccella* species, *R. fucoformis*, *R. hypomecha* and *R. linearis*, have been investigated. Besides considerable amounts

* Part VII. *Acta Chem. Scand.* **8** (1954) 1433.

of erythritol, small amounts of arabitol and mannitol were isolated, so in this respect the genus does not differ from the others of this order. An internal salt, choline sulphate, $(CH_3)_3\overset{+}{N}CH_2CH_2O\overline{S}O_3$, was also isolated from these lichens. Woolley and Peterson [2] isolated the same substance from the fungus *Aspergillus sydowi*, and this seems to be the only previous report of the isolation of this substance from natural material. As a lichen consists of an alga and a fungus, living in symbiosis, one can expect to find identical or similar substances in lichens and in fungi and choline sulphate is an example of a rather rare substance occurring in both groups. An investigation of isolated algae and fungi components of some lichens, would be of special interest from this point of view.

Two small fractions, containing a di- and a tri-saccharide, respectively, were also isolated from the *Roccella* lichens. These, on hydrolysis, yielded two reducing sugars, an aldose and a ketose which were chromatographically indistinguishable from galactose and tagatose. From the intensity of the spots, the proportions of aldose to ketose could be estimated as 1:1 and 2:1. Chromatographic evidence for the presence of the free sugars in the extracts was also obtained, and the galactose was isolated as its methylphenylhydrazone. Neither the tagatose nor the saccharides have as yet been obtained in a state of purity. The saccharides are very sensitive to hydrolysis and have probably been partly decomposed during the extraction and fractionation procedures. An epimerization, although improbable, is not excluded, and a further investigation of these saccharides is necessary. Tagatose has only been found once before in Nature, by Hirst, Hough and Jones [3] in the gum of *Sterculia setigera*. Galactose, glucose and mannose were the only sugars found in the hydrolysates of the extracted *Roccella* lichens.

EXPERIMENTAL

(Melting points uncorrected)

Dermatocarpon miniatum. The lichen (320 g) was extracted and the extract worked up as described in Part III[4]. Of the carbohydrate fraction (25 g) only a part (7 g) was fractionated on a carbon-Celite column, using the gradient elution technics, also described in Part III. The following substances were isolated: Mannitol (0.65 g), M. p. 161—162°; Volemitol (3.6 g), M. p. 149—150°; α,α-Trehalose (0.99 g), M. p. 96—100°; Sucrose (0.16 g), M. p. 178—180°. The melting points of all these compounds were not depressed on admixture with the corresponding authentic materials.

Roccella hypomecha. The lichen (90 g) was extracted and worked up as above. The carbohydrate fraction (5.8 g) was separated on a carbon column and the following substances were isolated:

Erythritol 2.3 g. M. p. 118—119°.
Choline sulphate 0.25 g. M. p. 310° (decomp.).
"Disaccharide" (amorphous) 0.25 g.
"Trisaccharide" (amorphous) 0.10 g.

The presence for arabitol and mannitol was indicated by means of paper chromatography.

Roccella linearis. The lichen (500 g) was extracted and worked up as above. The extract (59 g), was fractionated on a carbon column, using gradient elution with aqueous ethanol. The first fractions were shown to contain erythritol by paper chromatography but owing to mischance these fractions were lost before the amount of erythritol present

could be determined. Erythritol (2.9 g), m. p. 120—121°, and choline sulphate (0.8 g), m. p. 310° (decomp.), were isolated and in the mother liquor the presence of tagatose, galactose, arabitol and mannitol was indicated by paper chromatography. Crude fractions containing the disaccharide (0.8 g) and the trisaccharide (1.1 g) mentioned above, mixed with several unknown components, were also obtained.

The mother liquors from the erythritol and choline sulphate fractions were concentrated to a sirup (2.0 g) and fractionated on a hydrocellulose column (40 × 4.5 cm), using butanol saturated with water as solvent. Erythritol (0.28 g), m. p. 118—119°, arabitol (0.09 g), m. p. 99—100°, mannitol (0.05 g), m. p. 164—165°, and choline sulphate (0.04 g) were isolated. From one of the mother liquors galactose was isolated as its methylphenylhydrazone (0.05 g), m. p. 185—187°. The melting points of all these compounds were undepressed on admixture with the corresponding authentic materials. The ketose believed to be tagatose was found in the mother liquors from the arabitol but could not be separated from that substance. It gave colour reactions for ketoses, red with resorcinol-hydrochloric acid and yellow with anisidine phosphate. Fructose and tagatose have almost identical R_F-values in many solvent systems. In butanol-pyridine-water (3:1:1.5), however, they are separated, tagatose having the highest R_F-value of the two.

Roccella fucoformis. The lichen (300 g) was extracted as above, the methanol extract concentrated to dryness and treated with water (500 ml). Undissolved material was removed by filtration and the solution was deionized by stirring with a mixture of Ambelite IR 120 and IR 4 B, until the conductivity decreased to a constant value. The solution was then concentrated to a mixture of crystals and sirup and dissolved in boiling 90 % ethanol. On cooling crystals (7.5 g), m. p. 115—117°, separated. From the mother liquors a second crop (1.0 g) of the same m. p. was obtained. The combined crystalline fractions were refluxed with a mixture of acetic anhydride (100 ml) and pyridine (50 ml). Part of the material, which remained undissolved, was filtered off and recrystallised from ethanol, yielding pure choline sulphate (0.8 g), m. p. 308—317° (decomp.). The acetic anhydride-pyridine solution was concentrated to a sirup under reduced pressure and dissolved in hot ethanol. On cooling, the tetraacetate of erythritol (16.0 g), m. p. 85—87°, separated.

The mother liquors from the recrystallisation of the crude carbohydrate fraction were concentrated to a sirup (2.5 g) and fractionated on a hydrocellulose column as above. Erythritol (0.50 g), m. p. 116—118°, arabitol (0.18 g), m. p. 94—98°, mannitol (0.10 g), m. p. 163—164°, and choline sulphate (0.01 g), were isolated. In addition to these substances the presence of small amounts of galactose and tagatose in the mother liquors was indicated by means of paper chromatography. Crude fractions, containing small amounts of the di- and trisaccharide, were also obtained.

Choline sulphate. The "internal salt" isolated from the *Roccella* lichens was purified by recrystallisation from 90 % ethanol. It was first believed to be an ordinary salt, but later it was observed that it could not be removed from an aqueous solution by filtering through the ion exchange resins Amberlite IR 120 and IR 4 B. (Found: C 33.0; H 7.16; N 7.85; S 17.3. Calc. for $C_5H_{13}O_4NS$ (183.2): C 32.7; H 7.16; N 7.65; S 17.5).

Authentic material was prepared according to Schmidt [5] and was further purified by ion exchange and recrystallisation from 90 % ethanol. M. p. 310° (decomp.). The natural and the synthetic materials behaved identically on heating and a supersaturated solution of one of them immediately crystallised when seeded with the other.

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Oxidation of Δ^3-Carene

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The autoxidation of highly purified Δ^3-carene has been studied and oxidized samples have been analysed using a micro sorption method. The method has been found to be very sensitive and different types of sorptograms have been obtained.

One of the most well known properties of the terpene hydrocarbons is their tendency to autoxidatise. This quality of the technical turpentine is of fundamental importance when used for painting purposes, but on the other hand it can cause dermatological diseases on sensitive skin, especially that of house painters. Hellerström and Lundén [1] have shown the dermatitis to be in direct relation to the degree of oxidation of Swedish turpentine and in investigations by Hellerström, Thyresson, Blohm and Widmark [2,3] it was clearly demonstrated that only the products formed by the autoxidation are responsible for the skin activity.

In all work concerning allergy the chemicals used have to be purified to the highest degree, as even small amounts of impurities can give misleading results. In the case of terpenes it is difficult to obtain compounds free from isomers but we were kindly supplied, by Naval Stores Research Div., USA, with a sample of Δ^3-carene isolated from *Pinus ponderosa*, which, after steam distillation, gave no variations upon micro sorption analysis according to Blohm [4]. The choice of this terpene was favourable, as Δ^3-carene is a representative compound of the Swedish turpentine from *Pinus silvestris*. — All our samples of Δ^3-carene from Swedish sources gave great variations upon sorption analysis; compare the examples given in Fig. 1.

The oxidation of Δ^3-carene was studied earlier by Rau [5], who determined the rate of oxidation and showed it to be retarded by addition of pyrogallol and resorcinol. Owen and Simonsen [6] isolated *l*-1-methyl-3-*iso*propenyl-Δ^6-hexen-5-one from the oxidation products, and Fisher, Goldblatt *et al.*[7] found Δ^3-carene hydroperoxide to be an accelerator in the polymerization of rubber.

In this investigation Δ^3-carene was allowed to react with oxygen at room temperature in presence of water vapour in an apparatus shown in Fig. 2. At 11 intervals, regulated by the amount of oxygen consumed, samples were taken out and examined immediately. The samples were analysed by the

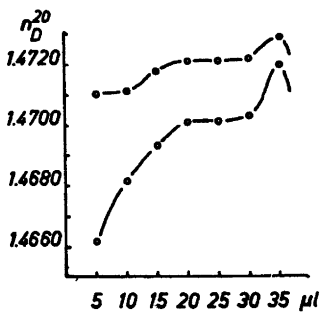

Fig. 1. Sorptograms of commercial samples of Δ^3-carene.

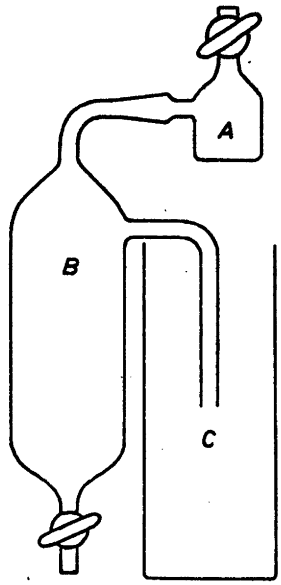

Fig. 2. Oxidation apparatus.

sorption method mentioned and there was found to be a marked change in the sorptograms even at the very first stage of the oxidation, thus demonstrating the sensitivity of the sorption method * (Fig. 3).

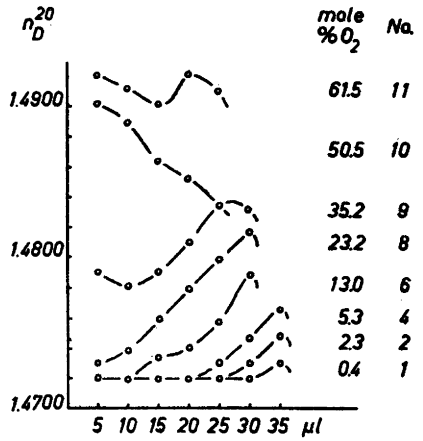

Fig. 3. Sorptograms of the oxidized samples.

* At some of the intervals mentioned samples were sealed carefully in small dark glass ampoules in an atmosphere of nitrogen and stored for two months. On sorption analysis there was a marked change over to the later stages of oxidation, thus the sorptograms of samples 6 and 9 in Fig. 3 after storing resembled those of 9 and 10. At the same time there was an increase in viscosity and a decrease in the peroxide number. — When some ampoules were opened a considerable overpressure was observed.

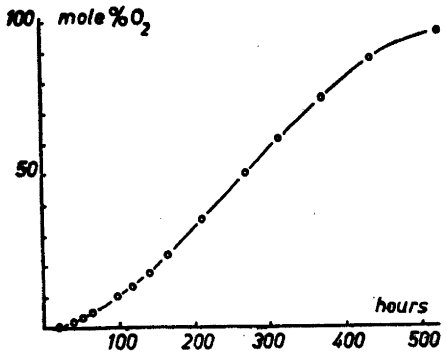

Fig. 4. Consumption of oxygen.

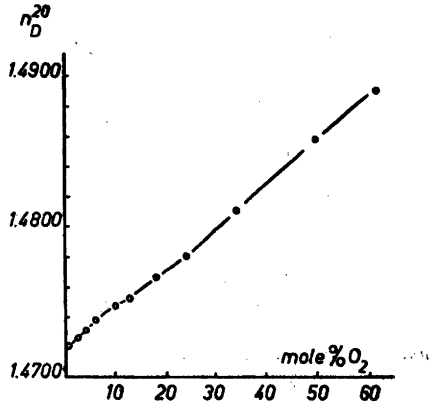

Fig. 5. Increase in refractive indices.

In connection with the sorption analysis, the viscosity and the content of the hydroperoxide were determined. As only a small amount of Δ^3-carene was available, these determinations were performed on a micro scale. This explains the choice of methods — the flow-time of a micro pipette, and iodine titrations, respectively. We realize the disadvantages in these two methods and the results obtained, especially those concerned with the later stages of the oxidation, cannot be considered to be entirely reliable.

In the oxidation, three stages can be observed in Fig. 4: a slow phase of initiation, a middle section with a fairly steady rate of reaction, and a retarded ending. — Fig. 5 gives the increase in refractive indices. — The phase of initiation, which corresponds to a consumption of up to 5—6 mole % oxygen — samples 1 to 4 — is characterized by a slow oxidation, a very slight increase in viscosity, a marked formation of peaks at the end of the sorptograms (Fig. 3) and a direct relation (2:1) between the oxygen consumed and that liberating iodine upon titration (Fig. 6). The dermatological activity is confined to the peak fractions [3]. The boundary between the terpene and the displacer has a typical odour of geranium.

After this initiation, the rate of oxidation accelerates and the consumption of oxygen is almost linear against time. The relation between consumed and liberated oxygen no longer holds and less iodine is liberated in the titrations; see experimental part. There is also a marked increase in the viscosity, especially at 30—40 mole % oxygen (*cf.* Fig. 7). The sorptograms give quite another figure. First the bottom of the sorptograms becomes wider and after 20 mole % of oxygen there is a sudden change with sample 6 where the first fractions have high refractive indices, and this figure characterizes the rest of the sorptograms. The samples now start to become mixed with the displacer, and even the sorption fractions taken at 50 μl give refractive indices above the value of ethanol. At 60 mole % of oxygen the liquid becomes opalescent and is too viscous to be analysed by sorption. However, the consumption of oxygen

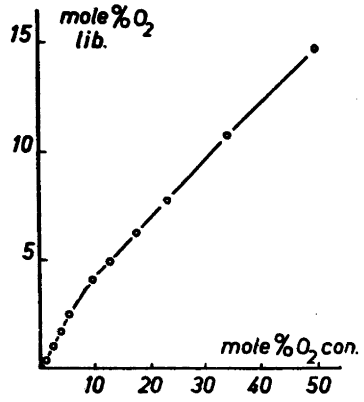

Fig. 6. Relation between oxygen consumed and that liberating iodine upon titration.

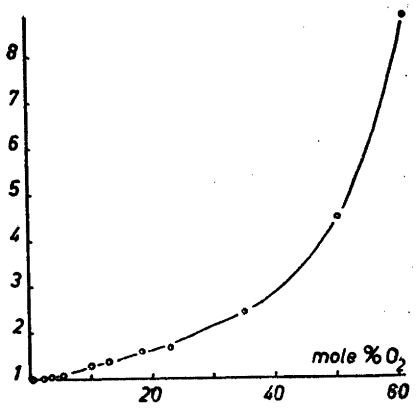

Fig. 7. Viscosity relative to pure Δ^3-carene.

has been followed to about 100 mole%. At 75 mole% a yellow colour is observed and the rate of oxidation is retarded. Then, the intensity of the colour increases and the last sample investigated was a strongly yellow resin.

EXPERIMENTAL PART

Oxidation. The Δ^3-carene used was supplied by Naval Stores Research Div., Olustee, Florida, USA, and had been isolated from ponderosa stump turpentine by vacuum distillation (100 TP, boiling point 65.5° C at 20 mm Hg). Immediately before the oxidation, the terpene was distilled with water vapour in the presence of pellets of sodium hydroxide. After separation, the liquid was dried over Na_2SO_4 and analysed. All treatments of the terpene were made in a nitrogen atmosphere.

The oxidation was carried out (in diffuse daylight) in an apparatus shown in Fig. 2. To the vessel *A* (attached to *B* with a long joint) was added 5 g of Δ^3-carene, which had a surface of 4.15 cm². The apparatus was filled with oxygen and the oxygen consumed by reaction with carene was replaced with water from container *C*, in such a way that the pressure remained almost constant. The temperature was controlled to 20° ± 2° C.

On analysis, the samples for both the chemical and physiological tests were removed, the vessel *A* being weighed before and after. The quantity of oxygen consumed was calculated on the amount of terpene mixture remaining. No correction was made for any water vapour which may have dissolved in the terpene.

Micro sorption analyses. The method devised by Blohm [4] was followed. 40 µl samples were separated into 5 µl fractions by displacement sorption on silica gel. To transfer the sample quantitatively to the column, the micro pipette was rinsed twice with 40 µl abs. ethanol. The column was 250 mm long and had an inner diameter of 1.4 mm. Absolute ethanol was used as displacer and the gel (Davison, 22 08 × 19 26, through 200 mesh) was activated 3—5 hours at 140° C, at 15 mm Hg. The refractive indices of the 5 µl fractions were determined according to Blohm [8] (refractometer Carl Zeiss, No. 70 617, accuracy ± 0.0001).

Viscosity. The micro pipette (40 µl), with marks above and below the bulb, was filled with the terpene sample and suspended vertically. The temperature was 22° ± 1°C. The time for the passage between the two marks was measured with a stop-watch, several drops being formed. The variations were found to be ± 0.2 seconds and the mean value from five readings was taken. The values given in Fig. 7 have been calculated

relative to pure Δ^3-carene, 9.0 ± 0.2 seconds. — With the same equipment the mean value for abs. ethanol was determined to be 9.6 ± 0.2 seconds.

Peroxide titrations. The determination of olefinic hydroperoxides using the iodine method has been investigated by several authors, and many similar techniques have been given. The possible addition of iodine to the double-bond, the influence of the sample size, and the effect of oxygen on the titration are sources of error — *cf.* Barnard and Hargrave [9] who have reported an accurate method, unfortunately too insensitive for our use on a micro scale. It is, however, generally accepted that the errors of the iodine titrations are reduced when performed on a micro scale — *cf.* Lampitt and Sylvester [10].

A ground joint flask, filled with nitrogen, was weighed before and after addition of a 40 µl test sample. 2.0 ml glacial acetic acid and 1.5 ml saturated potassium iodide solution were then added. After exactly 10 minutes, 2.0 ml water were added and the contents were titrated with 0.002 *N* thiosulfate solution (for the last three samples 0.004—0.006 *N*), using 0.05 ml starch solution. The thiosulfate solution was prepared immediately before use from 0.1 *N* solution. A blank was made for each titration. The procedure given is a micro modification of Yamada's [11] instructions. The results are given in Fig. 6.

Acknowledgement. The investigation has been supported by the *Swedish Medical Research Council.*

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Δ^3-Carene and α-Pinene from Swedish Sulfate Turpentine

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Δ^3-Carene and α-pinene from Swedish sulfate turpentine have been purified to index homogeneity (n_D^{25} = 1.4700 and 1.4632, respectively) and have been examined by an analytical micro sorption method. The extent of purification effected by vacuum distillation and methanol extraction has been investigated, and the conclusion drawn that only very efficient column distillation will give satisfactory results. α-Pinene is isomerized to a small extent on prolonged distillation but the resulting compounds can be removed by extraction.

The products which are formed by autoxidation of Δ^3-carene and α-pinene can easily be removed by extraction. This is also found to be the case when some oxygen-containing terpenes are mixed with the two terpenes, but it has been shown that small amounts of terpene hydrocarbons added are not affected by the extractions.

The industrial Swedish turpentine has been analysed by sorption and highly concentrated fractions of α-pinene, and Δ^3-carene are found in the first and the last fractions, respectively. The minor constituents in the α-pinene fractions are found to be Δ^3-carene and β-pinene and their content has been determined. The impurities in the Δ^3-carene fractions have been briefly studied.

The presence of β-pinene has been detected by catalytic Pd-$BaCO_3$ isomerization of β-pinene to α-pinene in connection with sorption analysis. The content of β-pinene in some fractions has been determined by adding known amounts of pure β-pinene to the isomerized samples to bring their sorptograms up to their original figures.

Most of the turpentine which is produced in Sweden is prepared at the sulfate pulp mills. At these mills the main source of wood is from *Pinus silvestris* but a smaller amount of wood from *Picea abies* L.* is almost always present, usually in the form of peeled waste from the sulfite mills. As this inferior wood is known to contain much less turpentine than the heart-wood of the pines [1], its influence on the composition of the sulfate turpentine is frequently neglected. It would, however, be interesting to investigate the turpentine prepared from unmixed wood of the two species.

* = *(Picea excelsa.)*

The turpentine is liberated with the steam which is blown off from the boilers at intervals in the heating up period, also during and after the sulfide boiling. Collected from the ring of boilers, the raw turpentine is freed, with the loss of some turpentine, from most of the low boiling sulfur compounds (a) which are burned mostly for the recovery of sulfur. The residue is usually distilled through a column and separated into three fractions; a strongly mercaptan-smelling foreoil (b), the turpentine (c) and a higher boiling fraction (d). The composition of these fractions from the charge of raw turpentine (17 000 l) used for the preparation of the turpentine for this investigation is given below *.

a) Fuel oil	1 150 l =	6.8 %
b) Fore oil	1 750 l =	10.3 »
c) Turpentine	8 500 l =	50.0 »
d) "Pine oil"	2 400 l =	14.1 »
e) Residue	2 700 l =	15.9 »
Loss	500 l =	2.9 »
	17 000 l =	100 %

The main constituents of the turpentine fraction from *Pinus silvestris* are known to be α-pinene and Δ^3-carene. The presence of β-pinene has been proved by the preparation of nopinonic acid [2,3], and Bardyshev *et al.*[4] have calculated the content in Siberian balsam turpentine to be 6.36 %. Aschan [2], and Semmler and Schiller [5] have presumed Δ^4-carene to be present but no proof has been given. When, however, discussing the minor constituents in turpentine, several difficulties must be kept in mind. Isomerization can have occurred during the preparation and purification operations. Azeotropic mixtures within a particular distillation fraction can be formed, and derivatives for characterization purposes are usually formed in low yields from terpene mixtures. Last but not least, until very recently there was no method for determination of the homogeneity of the liquid terpenes. An added complication is found in the case of sulfate turpentine which contains sulfur compounds. On the other hand, the alkaline sulfide is believed to have little effect on the isomerization of terpenes.

In this investigation, samples were taken out at intervals from an industrial distillation of the turpentine fraction c — see above. The samples were examined with the usual physical methods, before and after attempts to remove the residual mercaptan-smelling components. The smell was completely removed by storing the samples one week over sodium wire, during which time a brown precipitate was deposited. Alternatively, after five extractions with 90 % methanol, the smell was almost completely removed. As seen from Table 1, there was very little change in the physical constants after these treatments.

Examining the curves in Fig. 1, the three plateaux indicate that three components are present, but the physical values give, of course, no indication

* All the turpentine used in this investigation was kindly supplied by Marma-Långrör Ltd., Marmaverken. I am very much indebted for all help with the samples and industrial information to Ingenjör H. Jansson at this mill.

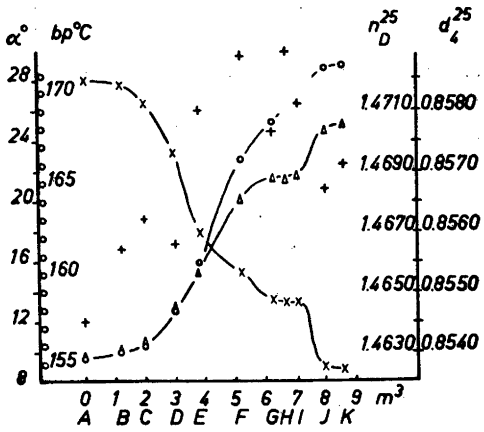

Fig. 1. Physical values of the fractions A—K from an industrial distillation of turpentine. × = optical rotation (10 cm). △ = refractive index. + = density. ○ = boiling point. The thermometer was unfortunately out of function for the fractions H and I.

of their homogeneity. However, in this work, the homogeneity of the turpentine fractions has been studied for the first time. The method used was devised by Blohm [6] and involves micro separation on activated silica gel. One drop (40.0 μl) was displaced with ethanol at pressure (1 atm) through a 25.0 cm long, narrow (i. d. 1.4 mm) column and 5 μl fractions were collected, on which refractive indices were determined [7]. The sorptogram, n_D^{25} plotted against sorption fraction number, will give a straight line for a homogeneous sample if no isomerization has been caused by the active gel.

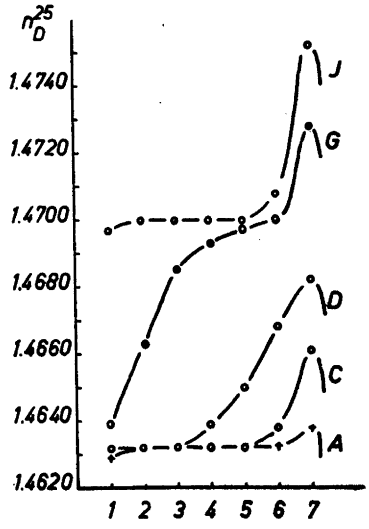

Fig. 2. Sorptograms of some of the fractions given in Fig. 1.

Table 1. *a) Sorptograms of industrial distillation of the turpentine fraction (c) at 758—760 mm Hg.*
b) Sorptograms after 5 methanol extractions.
c) Sorptograms after 2 weeks with sodium wire.
d) Sorptograms after Pd-$BaCO_3$ isomerization.
e) Sorptograms after addition of known amounts of pure β-pinene to the isomerized sample, fraction C 1 %, E 12 % and I 13 %.

Fraction	Refractive indices of sorptograms, n_D^{25} 1. . . . .
A	a) 4631_{US}* 4630_1 4632_{2-5} 4633_6 4638_7
	b) 4631_{US} 4632_{1-6} 4642_7
	c) 4632_{1-5} 4633_6 4641_7
	d) 4632_{US} 4627_1 4630_2 4632_{3-6} 4637_7
B	a) 4632_{US} 4630_1 4632_{2-6} 4643_7
	b) 4631_{US} 4632_{1-6} 4641_7
	c) 4632_{1-6} 4652_7
	d) $4632_{US,\ 1-6}$ 4650_7
C	a) 4636_{US} 4631_1 4632_{2-5} 4638_6 4661_7
	b) 4632_{US} 4632_{1-5} 4638_6 4661_7
	c) 4632_{1-5} 4638_6 4660_7
	d) 4633_{US} 4630_1 4632_{2-5} 4633_6 4649_7
	e) 4632_{1-6} 4659_7
D	a) 4648_{US} 4632_{1-3} 4638_4 4650_5 4668_6 4682_7
	b) 4650_{US} 4632_{1-4} 4635_5 4672_6 4700_7
	c) 4632_{1-4} 4638_5 4653_6 4685_7
	d) 4640_{US} 4628_1 4631_2 4632_{3-5} 4642_6 4670_7
E	a) 4662_{US} 4632_{1-3} 4647_4 4670_5 4690_6 4704_7
	b) 4662_{US} 4632_{1-3} 4644_4 4680_5 4693_6 4706_7
	c) 4632_{1-3} 4634_3 4650_4 4677_5 4690_6 4700_7
	d) 4650_{US} 4630_1 4632_2 4633_{3-4} 4642_5 4668_6 4682_7
	e) 4629_1 4631_2 4642_3 4658_4 4678_5 4690_6 4702_7
F	a) 4684_{US} 4638_1 4653_2 4677_3 4689_4 4697_5 4700_6 4710_7
	b) 4683_{US} 4632_1 4652_2 4678_3 4689_4 4698_5 4700_6 4713_7
	c) 4632_1 4646_2 4675_3 4688_4 4692_5 4698_6 4712_7
	d) 4682_{US} 4632_1 4653_2 4672_3 4686_4 4690_5 4710_6
G	a) 4690_{US} 4645_1 4666_2 4682_3 4691_4 4698_5 4700_6 4722_7
	b) 4689_{US} 4640_1 4663_2 4685_3 4693_4 4698_5 4700_6 4728_7
	c) 4638_1 4668_2 4683_3 4692_4 4700_{5-6} 4721_7
	d) 4698_{US} 4642_1 4663_2 4685_3 4695_4 4699_5 4703_6 4753_7
H	a) 4690_{US} 4646_1 4667_2 4682_3 4691_4 4700_{5-6} 4722_7
	b) 4690_{US} 4647_1 4670_2 4688_3 4697_4 4699_5 4708_6 4730_7
	c) 4641_1 4661_2 4680_3 4692_4 4698_5 4700_6 4718_7
	d) 4690_{US} 4648_1 4672_2 4689_3 4692_4 4700_5 4703_6 4742_7
I	a) 4684_{US} 4637_1 4653_2 4682_3 4693_4 4700_{5-6} 4710_7
	b) 4688_{US} 4637_1 4661_2 4683_3 4693_4 4700_{5-6} 4729_7
	c) 4632_1 4654_2 4679_3 4689_4 4692_5 4700_6 4714_7
	d) 4671_{US} 4627_1 4640_2 4662_3 4678_4 4682_5 4688_6 4703_7
	e) 4626_1 4640_2 4669_3 4688_4 4693_5 4698_6 4704_7
J	a) 4709_{US} 4697_1 4700_{2-5} 4710_6 4752_7
	b) 4710_{US} 4693_1 4699_2 4700_{3-5} 4711_6 4753_7
	c) 4692_1 4698_2 4700_{3-5} 4708_6 4748_7
	d) 4708_{US} 4681_1 4692_2 4693_{3-4} 4695_5 4710_6 4756_7
K	a) 4710_{US} 4700_{1-5} 4709_6 4748_7
	b) 4710_{US} 4700_{1-4} 4701_5 4717_6 4757_7
	c) 4696_1 4700_{2-5} 4708_6 4747_7
	d) 4710_{US} 4697_1 4700_{2-5} 4705_6 4748_7

* see p. 935, note **.

With the aid of this method it was shown (*cf.* Table 1 and Fig. 2) that the first plateau consisted of a rather homogeneous α-pinene, the last plateau was a somewhat less pure Δ^3-carene and the middle one was a more complex mixture. At every sorption analysis the last fractions had higher refractive indices and obviously contained terpenes of more complex nature, thus giving the sorptogram a peak before the fall to the ethanol level. For α-pinene, the obvious explanation is that small amounts of Δ^3-carene and β-pinene are present in the mixture (see below), but in the case of Δ^3-carene there is no simple explanation. To study the problems concerning this subject it is necessary to find methods for the purification of other terpenes which can be presumed to be present in the mixture, either as natural compounds of the wood, or formed by isomerization during the sulfide boiling or on oxidation. Furthermore, the general conditions and the sensitivity of the analytical method cannot be examined until more terpenes have been purified to index homogeneity.

Purification of Δ^3-carene

In agreement with Rao and Simonsen [3] on their investigation of Swedish turpentine, the purest Δ^3-carene was found in the last fractions of the distillation. To find the best raw material for the purification of Δ^3-carene, samples in the region of sample K from several industrial distillations were examined. There was a rather wide distribution of the sorptograms with different heights of the peaks. The last fractions were without any slope of α-pinene in the beginning of the sorptograms, but as the composition of the "pine oil" (d) is rather uncertain (compare Rao and Simonsen [3] who have isolated *l*-cadinene) a fraction containing some percent α-pinene was used as starting material for the purification. After standing for two weeks over sodium, the brown

Table 2. Distillation of 1.5 l Δ^3-carene, sample K_3, in a laboratory column (a) at 13 mm Hg. Boiling temperature 55.8—57.4°. Reflux ratio 14:15. Time 85 h.

Fraction No.	Volume ml	Refractive indices of sorptograms, n_D^{25} 1. ....
1	50	4691_1 4694_2 4697_{3-4} 4700_5 4703_6 4728_7
2	50	4692_1 4697_2 4700_{3-5} 4708_6 4735_7
3	50	4695_1 4698_2 4700_{3-5} 4708_6 4734_7
4	50	4706_{US} 4700_{1-4} 4701_5 4712_6 4726_7
5	50	4700_{1-4} 4702_5 4708_6 4729_7
6—7	100	4703_{US} 4700_{1-4} 4702_5 4705_6 4728_7
8	50	4700_{1-4} 4702_5 4706_6 4725_7
9	50	4700_{1-4} 4701_5 4703_6 4728_7
10	50	4700_{1-6} 4703_7 4729_8
11—19	525	$4702\text{-}3_{US}$ 4700_{1-6} $4727\text{-}30_7$
20	50	4704_{US} 4700_{1-6} 4738_7
21	50	4707_{US} 4700_{1-5} 4702_6 4745_7
22	50	4709_{US} 4703_{1-2} 4704_{3-4} 4710_5 4732_6 4763_7
(23)—24	100	4732_{US} 4702_1 4703_2 4713_3 4730_4 4748_5 4763_6 4771_7
25	35	4758_{US}
Residue	120	4772_{US} 4761_{1-2} 4770_3 4782_4 4792_5 4797_6 4775_7

Table 3. Distillation of 35 ml Δ^3-carene, sample K_2, in a spinning band column [14] at 20 mm Hg. Fractions 2 to 10 boiling at 70.0°.

Fraction No.	Volume total %	Refractive indices of sorptograms, n_D^{25} 1. . . . .
1	7.5	4707_{US} 4688_1 4697_2 4700_{2-5} 4705_6 4728_7
2	15	4709_{US} 4698_1 4700_{2-5} 4702_6 4736_7
3	22.5	4706_{US} 4698_1 4700_{2-6} 4732_7
4	30	4703_{US} 4698_1 4700_{2-6} 4730_7
5	38	4703_{US} 4698_1 4700_{2-6} 4708_7 4732_8
6—7	53	4704_{US} 4698_1 4700_{2-5} 4726_7
8	60.5	4704_{US} 4699_1 4700_{2-5} 4736_7
9	68	4708_{US} 4700_{1-6} 4748_7
10	75.5	4710_{US} 4700_{1-5} 4709_6 4765_7
11	83.5	4711_{US} 4700_{1-5} 4726_6 4798_7
12	89	4736_{US} 4700_{1-3} 4722_4 4757_5 4779_6 4803_7

deposit was filtered off and the liquid was used in the next purification stage. Further treatment with sodium gave no more precipitate, unless the sample became oxidized in the meantime.

In order to remove the most hydrophilic components — after vacuum distillation — the liquid was shaken several times with 10 % of its volume of 90 % methanol. This treatment had an effect on the peak, and the first extractions reduced the peak more than the subsequent ones. After 60—70 extractions there was no further reduction, and the peak remained constant with 9 units in the last figure above the level. As carene is somewhat soluble in 90 % methanol only a very small amount of the terpene was left after all these extractions.

The only way found to purify Δ^3-carene to index homogeneity was by vacuum distillation using a fractionating column. An ordinary laboratory column, 40 cm high, with reflux ratio 14:15 and a spinning band column [8] both operating in vacuum, gave no marked reduction in the peak (Tables 2 and 3). When distilled up a 6′ high Podbielniak column, there was a consider-

Table 4. Distillation of 2.0 l Δ^3-carene, sample K_2, in a 6′ Podbielniak column (e) at 19.5 mm Hg. Fractions 4 to 24 boiling at 66.5°. Reflux ratio fractions 1—8, 199:200; fraction 9—24, 299:300. Time 24 days.

Fraction No.	Volume total %	Refractive indices sorptograms, n_D^{25} 1.....	Optical rotation (10 cm)
5	17.5	4699_{US} 4693_1 4696_2 4698_3 4700_{4-6} 4708_7	13.28°
6	22.5	4699_{US} 4700_{1-6} 4708_7	13.73°
7—13	45	4699_{US} 4700_{1-6} $4705-3_7$	(10) 13.84°
14	48.5	4700_{US} 4700_{1-6} 4703_7	14.49°
15—16	55.5	4700_{US} 4700_{1-6} 4701_7	(15) 14.80°
17—19	65	4700_{US} 4700_{1-7}	(18) 15.22°
20—22	79.5	4700_{US} 4700_{1-6} 4702_7	(20) 15.27°
23	82.5	4700_{US} 4700_{1-6} 4703_7	(22) 15.25°
24	85.5	4700_{1-5} 4707_6 4763_7	12.78°

able reduction, but index-homogeneous fractions were obtained only when the reflux ratio was raised to 299:300 (equal to 100—140 TP). As seen from Table 4, most fractions, in spite of the high efficiency of the column, had small peaks and it was only found possible to reduce them a little by methanol extraction.

The physical constants found for index-homogeneous Δ^3-carene are $[\alpha]_D^{25} = +17.6°$; $d_4^{25} = 0.8635$; $n_D^{25} = 1.4700$; as the refractive index by the sorption analysis has become the most important constant it has been determined at different temperatures giving a decrease of 0.0023 per 5° in the range of +15° to +30°.

The nature of the peak constituents has been briefly investigated, but a closer study cannot be made until the supposed constituents have been prepared to index homogeneity. From the sorptograms of mixtures of the two terpenes [9] β-pinene and *d*-limonene, purified to index homogeneity, it can be seen, that they can be almost excluded as peak constituents. When pure carene was mixed with 5 % of inhomogeneous — in spite of vacuum distillation — α-terpinene, terpinolene and "sylvestrene", peaks were obtained, which did not change after methanol extractions. The peak which is formed when Δ^3-carene is oxidized (*cf.* Blohm and Widmark [10]) is easily removed by 2—3 extractions. If, however, the oxidized carene is stored in a sealed glass ampoule for several months, the nature of the peak changes, and becomes as difficult to remove as in the case of the industrial sample.

Purification of α-pinene

Several industrial charges of turpentine were examined and an α-pinene fraction (A_6) with a very low peak was selected for further purification. After the first few extractions with methanol, or after sodium treatment, there was an increase in the height of the peak. This was most probably due to the removal of some hydrophilic sulfur compounds with low refractive index. Further extractions gave no changes in the peak, even after 60 extractions.

Vacuum distillation using the laboratory column (Table 5) gave a reduction of the peak, and with a higher column the peak remained only in the first

Table 5. Distillation of 405 ml α-pinene, sample A_3, in a laboratory column (a) at 15.0 mm Hg. Boiling temperature 46.1°—46.7°. Reflux ratio 17:18. Time 20 h.

Fraction No.	Volume ml	Refractive indices of sorptograms, n_D^{25} 1. ....
1	20	4630_{US} 4629_1 4631_3 4632_{3-5} 4634_6 4637_7 4657_8
2	30	4630_{US} 4631_1 4632_{3-5} 4637_6 4650_7
3	30	4629_{US} 4632_{1-5} 4635_6 4645_7
4	30	4630_{US} 4632_{1-5} 4634_6 4644_7
5—7	90	4631_{US} 4632_{1-5} 4634_6 4644_7
8—9	60	4631_{US} 4632_{1-5} 4635_6 4645_7
10	30	$4632_{US,\ 1-4}$ 4637_5 4640_6 4656_7
11	20	$4632_{US,\ 1-3}$ 4634_4 4639_5 4644_6 4662_7
Residue	75	4670_{US} 4642_1 4653_3 4663_3 4672_4 4685_5 4692_6 4690_7

Table 6. Distillation of 275 g α-pinene, sample A_6, in a 2 m column (d) (60 mm Hg). Boiling temperature 77.0°–77.5°. Reflux ratio 24:25. Time 12 h.

Fraction No.	Volume ml	Refractive indices of sorptograms, n_D^{25} 1. . . . .
1	41.1	4628_1 4629_2 4630_{3-5} 4632_6 4633_7
2	35.7	4629_{US} 4632_{1-7}
3	59.0	4629_{US} 4632_{1-7}
4 *	11.7	4629_{US} 4632_{1-7}

* By accident no more fractions were obtained.

of 4 fractions (Table 6). However, there was a difference in refractive index between the unsorbed sample and the plateau in the sorptogram; $n_D^{25} = 1.4629$ and 1.4632, respectively, probably depending on compounds of low index which were mixed up with the displacer during the sorption. Distilled through the Podbielniak column all fractions had small peaks (Table 7), most likely formed by isomerization on prolonged distillation. Methanol extractions removed these peaks, and after washing and drying, the plateau and the unsorbed sample both had the same refractive index 1.4632.

The values found for α-pinene are

$$[\alpha]_D^{25} = +\ 34.9°;\quad d_4^{25} = 0.8571;\quad n_D^{25} = 1.4632$$

with a decrease of 0.0023 per 5° in the range of +15° to 30°. The value for the rotation is lower than those reported for *e. g.* Greek *d*-α-pinene. This could be explained by the content of *l*-α-pinene in our turpentine resulting from contamination of the pine wood by fir wood. However, α-pinene extracted from American wood [11] gives a rotation similar to ours.

In agreement with the findings in the case of Δ^3-carene, oxygen compounds added to pure α-pinene could be removed by methanol extractions, but the peaks which were formed by adding small amounts of Δ^3-carene and β-pinene were almost unaffected by extractions. Sorptograms similar to the ones for fractions A to C were formed when sorbing mixtures [9] of pure α-pinene with small amounts of Δ^3-carene or β-pinene; fraction A corresponded to 0.4 % Δ^3-carene or 1 % β-pinene, B to 1.5 % or 3 % and C 2 % or 5 %, respectively.

Table 7. Distillation of 800 ml α-pinene, sample A_6, in a 6′ Podbielniak column (e) (42.1–42.6 mm Hg). Boiling temperature 68.0–68.5°. Reflux ratio 299:300.

Fraction No.	Volume total %	Refractive indices of sorptograms, n_D^{23} 1. . . . .
1	7	4628_{US} 4623_1 4630_2 4631_3 4632_{4-7}
2	14	4629_{US} 4628_1 4630_2 4632_{3-7}
3	25	4630_{US} 4632_{1-6} 4635_7
4	32.5	4631_{US} 4632_{1-6} 4637_7
5–9	77	$4632_{US,\ 1-6}$ $4636-8_7$

As seen from the following paragraph, the peaks consisted of a mixture of the two terpenes, and the numbers above thus give the maximum content in the peaks.

Presence of β-pinene

Bardyshev *et al.*[4] have calculated the content of β-pinene in Siberian turpentine to be about 6 %. They have identified β-pinene by oxidation to nopinonic acid and explain earlier failures to detect β-pinene by demonstrating the difficulties in preparing nopinonic acid from β-pinene mixtures. To overcome this difficulty, an indirect method, catalytic conversion of β-pinene, was chosen for this investigation.

Richer and Wolff[12] have reported the catalytic isomerization of β-pinene to α-pinene by palladium-black in ether solution in the presence of hydrogen. Here, the more practical Pd-$BaCO_3$-catalyst was used without any solvent, on a half micro scale. In a following communication[13] clear evidence will be given to show that this reaction is quantitative, and that the reaction is almost always followed by a little hydrogenation and a second isomerization, which are found to be low in the case of α-pinene and Δ^3-carene. However, these side reactions hamper the interpretation of the sorptograms and smaller amounts than, *e. g.*, 1 % β-pinene in α-pinene cannot usually be detected. Furthermore, the activity of the catalyst and its ability to cause side reactions with other participating terpenes has to be investigated before each analysis.

One ml samples of the fractions A to K were shaken with about 50 mg of a 5 % Pd-$BaCO_3$ catalyst in small stoppered test tubes filled with hydrogen ($\sim$ 2 ml gas). The time, usually 1 to 2 hours, for full conversion was determined by simultaneous tests with index-homogeneous β-pinene. The presence of β-pinene or, more improbably, terpenes reacting in the same way, could be detected in the fractions B to I (*cf.* Table 1). For the fraction J, a sorptogram was obtained which differs from any isomerization tests with mixtures of Δ^3-carene and α- and β-pinene, and gives indications of more terpenes in our turpentine mixture.

In order to calculate roughly the content of β-pinene, known amounts of this terpene in index-homogeneous form were added to some of the isomerized samples to bring their sorptograms up to the original figures. From these experiments the content of β-pinene was found to be: fraction C 1—2 %, E 12 %, and I 13 %. Comparing the curves after isomerization with those from α-pinene-Δ^3-carene mixtures[9] one can calculate the content of Δ^3-carene to be in fraction B and C 1 %, D 3 %, and E 5 %.

Investigation of the middle fractions

The figures from the sorptograms of the fractions E to I differed from those obtained by mixing index-homogeneous Δ^3-carene and α- and β-pinene[9], not only in presence of peaks but also by the slope of the curves. After isomerization, there was still a difference which could not be explained only by the side reactions of the compounds mentioned. It is, of course, difficult to obtain perfectly reproducible sorptograms here with such steep curves as, *e. g.*, small variations in the 5 μl volumes can cause considerable variations in

Table 8. Distillation of sample H (150 ml) in a laboratory column a at 756 mm Hg. Reflux ratio 15:16. Time 4.30 h. 15 ml fractions were collected.

Fraction No.	Temp. °C	Refractive indices of sorptograms, n_D^{25} 1. . . . .
1	162.1	4662_{US} 4627_1 4632_{2-3} 4651_4 4675_5 4691_6 4707_7
2	162.9	4662_{US} 4628_1 4630_2 4632_3 4659_4 4678_5 4696_6 4702_7
3	164.5	4670_{US} 4630_1 4633_2 4647_3 4668_4 4684_5 4698_6 4707_7
4	165.4	4678_{US} 4630_1 4648_2 4671_3 4689_4 4699_5 4704_6 4718_7
5	167.0	4688_{US} 4640^1 4650_2 4673_3 4682_4 4692_5 4700_6 4709_7
6	168.8	4691_{US} 4656_1 4675_2 4689_3 4699_4 4700_5 4704_6 4719_7
7	170.4	4700_{US} 4670_1 4689_2 4700_3 4704_4 4708_5 4728_6
8	170.0	4702_{US} 4688_1 4700_{2-4} 4703_5 4705_6 4728_7
9	170.0	4708_{US} 4690_1 4700_{2-5} 4705_6 4740_7
Residue		4760_{US}

the index values. However, the discrepancy given above cannot be explained by non-reproducibility of the sorptograms, as from experience these are found to be much more reproducible. This gives additional evidence for more terpenes being present in the turpentine. Owing to the discrepancy it is not found possible to calculate, by comparison, the content of Δ^3-carene in the fractions F to J.

In order to examine briefly the presence of other terpenes, fraction H (the middle plateau in Fig. 1) was distilled through a column both at atmospheric pressure and under vacuum. The very poor separation at ordinary pressure (Table 8) gave an indication of an azeotropic mixture. On vacuum distillation (Table 9) there was a separation into two fractions of almost pure α-pinene, a middle fraction and finally two fractions (H_4 and H_5) which from their sorptograms could be Δ^3-carene with small amounts of β-pinene and α-pinene. However, when the β-pinene was removed by isomerization, sorptograms

Table 9. a) Distillation of sample H (50 ml) in a 60 cm column (c) at 10—12 mm Hg. Reflux ratio 9:10. Time 4 h. b) Sorptograms after Pd-$BaCO_3$ isomerization for 3 hours.

Fraction No.	Volume ml	Temp. °C	Refractive indices of sorptograms, n_D^{25} 1. . . . .
1	5.6	40.0	a) 4634_{US} 4629_1 4631_2 4632_{3-6} 4646_7 b) 4632_{1-5} 4633_6 4648_7
2	7.1	43.8	a) 4636_{US} 4632_{1-5} 4638_6 4663_7 b) 4632_{1-5} 4640_6 4664_7
3	5.5	51.0	a) 4654_{US} 4632_1 4642_2 4661_3 4687_4 4702_5 4712_6 4722_7 b) 4632_1 4641_2 4662_3 4691_4 4707_5 4713_6 4718_7
4	6.0	52.7	a) 4712_{US} 4700_1 4707_2 4709_3 4712_4 4714_{5-7} b) 4695_1 4708_2 4710_3 4711_{4-6} 4717_7
5	3.0	53.1	a) 4710_{US} 4699_1 4703_2 4705_3 4708_4 4710_5 4711_6 4714_7 b) 4693_1 4702_2 4704_{3-5} 4705_6 4709_7
Residue	18		a) 4718_{US} 4709_1 4703_2 4700_{3-6} 4724_7 b) 4700_{1-6} 4755_7

were obtained which were different from those from α-pinene-Δ^3-carene mixtures [9]. This discovery will be further investigated with aid of a more effective vacuum column.

EXPERIMENTAL PART

Equipment

Refractometer, Bellingham & Stanley No. 402330. Calibrated against standard plate n_D 1.5009. A thermostat controlled the temperature to 25 ± 0.02° C.

Polarimeter, Bellingham & Stanley No. 36291, 20.0 cm tubes being used. All values are calculated for 10 cm.

Pycnometers. Two types were used: Ostwald-Sprengel (type E) [14] for the more accurate determination of the pure terpenes and a usual density bottle for the industrial samples. The procedure given by Bauer [14] has been followed.

Sorption apparatus. An apparatus, fully observing Blohm's instructions, was used. The silica gel (Davison, 922—08—226 through 200 mesh) was activated 2 hours at 15 mm Hg. On reading the indices the aluminium setting [7] was omitted, and a fresh paper (~ 5 × 5 mm) was used for each determination.

Columns. a) 40 cm laboratory column resembling Quickfit No. FC 11/23 filled with 3 mm Fenske steel helices. Refluxhead Quickfit No. FC 15/12 and Thiele receiver; b) Spinning-band column (*cf.* Björkman and Olavi [8]); c) 60 cm column (i. d. 17 mm) electrically compensated, filled with Fenske glass helices, Quickfit No. FC 8/45. Refluxhead modified Quickfit No. FC 15/122. Thiele receiver; d) 2 m column (i. d. 25 mm) filled with steel springs, circulating liquid compensation. Electronic receiver; e) Podbielniak column, Hyper-Cal Apparatus, Cat. No. HC-701-A, Ser. "D", 6′ high, inner diameter 25 mm *. All columns were filled with an inert gas, nitrogen or carbon dioxide, before the distillations and the fractions were changed without disturbing the vacuum.

Experiments

One liter samples were taken out at the intervals given in Fig. 1, the outlet tube first being cleaned by tapping out several liters of the corresponding turpentine. The samples were stored and transported in full, air free, bottles.

For selecting the best industrial sample of Δ^3-carene and α-pinene for purification and synthetic work, carboys of 30 liter capacity were filled at about 300 liters intervals in the region under investigation from several charges. A few ml from each carboy were sent for analysis. The best sample was then sent in tin containers to the laboratory where it was poured immediately into glass bottles. Sodium wire (d. 0.2 mm) — about 2 g per liter — was pressed into the liquid and the samples stored air tight in a dark cellar.

The sorptograms **, after Na-treatment of the used samples were, *Δ^3-carene* (K_3) 1.4706_{US} 1.4698_1 1.4700_{2-5} 1.4716_6 1.4750_7, *α-pinene* (A_6) 1.4630_{US} 1.4632_{1-5} 1.4633_8 1.4638_{7}, and (A_3) $1.4632_{US, 1-4}$ 1.4634_5 1.4639_6 1.4645_7 for a preliminary investigation.

Methanol extractions

Δ^3-Carene, 25 ml of fraction 11—19 (Table 2), was shaken 3 minutes with about 10 % of its volume with 90 % methanol in a joint test tube ***. After separation (15—30 minutes) the methanol layer was suctioned off and some of the loss of turpentine can be explained by emulsion formation. After 70 extractions only 3 ml were left.

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* The two distillations with this column have been performed by Mo and Domsjö, Ltd., Örnsköldvik. I am very much indebted to Dr. B. Weibull and Mr. B. Nycander for all help with the distillations.

** In order to simplify the printing of the sorptograms and to avoid too many printed figures the n_D^{25} values of the sorption are given with the fraction number as index. The value of the unsorbed sample is given US as index.

*** The minimal amount of grease was used, and 1 % grease in pure Δ^3-carene gave no change of the sorptogram.

7 extractions	1.4700_{1-5}	1.4702_6	1.4726_7
23 »	1.4700_{1-6}	1.4721_7	
33 »	1.4700_{1-6}	1.4715_7	
45 »	1.4700_{1-6}	1.4711_7	
55 »	1.4700_{1-6}	1.4710_7	
70 »	1.4700_{1-6}	1.4709_7	

Δ^3-Carene, 6 ml of fraction 7 (Table 4), was extracted 15 times which brought the peak down to 1.4702. Extracting fraction 14 (peak 1.4703) a peak of 1.4702 remained even after 20 shakes.

Freshly oxidized Δ^3-carene. 3 ml of index-homogeneous carene was stored 2 days in an open flask giving the sorptogram a peak, due to oxidation: 1.4712_{US} 1.4700_{1-5} 1.4712_6 1.4758_7. After 3 extractions the peak was fully removed.

Stored, oxidized Δ^3-carene. A 4 ml sample of pure carene was oxidized by accident, before it was sealed in an ampoule. After storing for several months it gave the sorptogram: 1.4701_{US} 1.4700_{1-6} 1.4709_7. When extracted, the peak was reduced with difficulty.

6 extractions	1.4700_{1-6}	1.4706_7
12 »	1.4700_{1-6}	1.4703_7

α-Pinene. 75 ml α-pinene, sorptogram 1.4632_{US} 1.4632_{1-5} 1.4639_6 1.4646_7, was extracted 30 times with 90 % methanol but there was no change in the peak. The same negative result was obtained by extraction with 50 % ethanol.

α-Pinene, obtained on prolonged vacuum distillation (fraction 3, Table 7) was extracted 5 times and gave then the sorptogram $1.4632_{US, 1-7}$.

Freshly oxidized α-pinene. 2 ml index-homogeneous α-pinene, stored in an open flask for 5 days, gave the sorptogram 1.4632_{1-4} 1.4636_5 1.4639_6 1.4642_7. Three methanol extractions restored the α-pinene to index homogeneity.

Extraction of mixtures

Δ^3-Carene and β-pinene in α-pinene. One percent Δ^3-carene in α-pinene and 1 % β-pinene in α-pinene gave the sorptograms 1.4632_{1-5} 1.4635_6 1.4649_7 and 1.4632_{1-5} 1.4634_6 1.4649_7, respectively. There was very little change after 30 extractions.

Verbenone in α-pinene. Two percent of carefully vacuum distilled verbenone; sorptogram: 1.4818_1 1.4850_2 1.4863_3 1.4869_4 1.4874_{5-6} 1.4672_7 were added to pure α-pinene forming the sorptogram $1.4632_{US, 1-6}$ 1.4645_7. The peak was removed after 10 extractions.

α-Terpinene, terpinolene, and "sylvestrene" in Δ^3-carene. Up to now there has been no success in purifying the available amounts of these terpenes to index homogeneity. After vacuum distillation the terpenes had the following sorptograms:

α-Terpinene 1.4760_{US} 1.4747_1 1.4752_2 1.4770_3 1.4781_4 1.4783_5 1.4784_6 1.4785_7;
Terpinolene 1.4757_{US} 1.4791_1 1.4797_2 1.4800_3 1.4802_{4-5} 1.4803_6;
"Sylvestrene" 1.4810_{US} 1.4856_1 1.4848_2 1.4837_3 1.4825_4 1.4803_5 1.4802_6 1.4793_7

Five percent of these samples in pure carene gave the sorptograms:

α-Terpinene: 1.4703_{US} 1.4700_{1-4} 1.4701_5 1.4715_6 1.4729_7;
Terpinolene 1.4704_{US} 1.4703_{1-2} 1.4701_{3-4} 1.4700_{5-6} 1.4720_7;
"Sylvestrene" 1.4708_{US} 1.4700_{1-3} 1.4705_4 1.4713_5 1.4722_6 1.4738_7.

After 20 methanol extractions of these mixtures there were no marked changes in the sorptograms.

α-Terpineol in Δ^3-carene. α-Terpineol was only very slowly forced through the column giving the sorptogram: 1.4800_{US} 1.4805_1 1.4798_2 1.4780_3 1.4720_4 1.4632_5. In 5 % mixture the sorption also proceeded slowly giving $1.4700_{US, 1-5}$ 1.4698_6 1.4684_7. Three extractions gave $1.4700_{US, 1-7}$.

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Purification of *d*-Limonene and *β*-Pinene

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By methanol extraction *d*-limonene and *β*-pinene have been purified to index homogeneity (n_D^{25} = 1.4703, 1.4762 respectively) when analysed for purity using a micro sorption method. *l*-Limonene, isolated from cones of *Abies alba* and purified on a micro scale gave the same sorptogram as *d*-limonene 1.4703$_{US\,1-7}$ and the same sorptogram as dipentene prepared by mixing the antipodes.

In an earlier communication [1] the author has studied the purification of Δ^3-carene and *β*-pinene from Swedish turpentine. It was found that only a combination of prolonged column distillation under vacuum and methanol extraction gave index-homogeneous products when analysed for purity with a micro sorption method devised by Blohm [2].

In this investigation, commercial samples of *d*-limonene and *β*-pinene were purified to index homogeneity and it was found that only one of the methods mentioned above, methanol extraction, was needed to bring these samples to index homogeneity.

The physical values for the two index homogeneous terpenes are as follows:

	$[\alpha]_D^{25}$	d_4^{25}	n_D^{25}
d-limonene	+ 123.3°	0.8409	1.4703
β-pinene	− 21.5°	0.8684	1.4762

In the range of +15° C to 30° C there is a decrease in refractive index of 0.0023 per 5° C.

Several attempts to reach the same stage of purity with samples of dipentene have been unsuccessful. An indirect way was chosen to study the eventual isomerization of dipentene caused by the active gel which is used in the analyses. Cones of *Abies alba* Mill. were extracted and a small amount of a turpentine was obtained, too small to distil through an efficient vacuum column. On displacement chromatography of 120 *μ*l of the extract, about 40 *μl* index-homogeneous *l*-limonene were obtained. When mixed with *d*-limonene the dipentene obtained was shown to be index-homogeneous on sorption analysis, thus demonstrating that the purification of dipentene can be followed with the analytical sorption method.

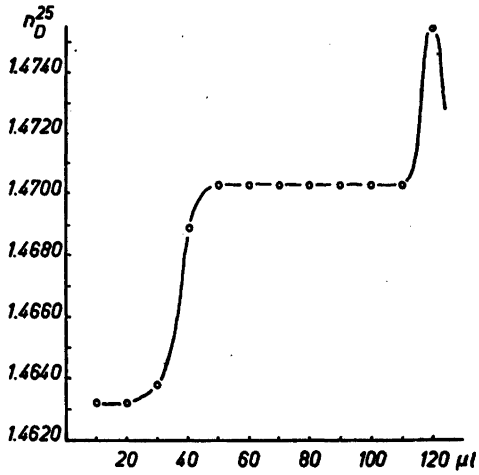

Fig. 1. Turpentine from cones of Abies alba *separated by displacement chromatography.*

EXPERIMENTAL PART

A micro sorption apparatus (column 250 × i. d. 1.4 mm), fully conforming to Blohm's instructions, was used. The silica gel (Davison, 922, 08, 226, through 200 mesh) was activated for 2 hours at 140° and 15 mm Hg. The refractometer, Bellingham & Stanley No. 402330, was calibrated against a standard plate n_D 1.5009. The thermostat was controlled to 25° ± 0.02°. For reading the refractive indices, a fresh paper ~ 5 × 5 mm was used for each determination.

The optical rotations were measured without any solvent in 20 cm tubes; polarimeter Bellingham & Stanley No. 36291. The densities were determined according to Bauer [3], using Ostwald-Sprengel pycnometers with small cups (type E).

Commercial samples of *d*-limonene (Eastman, Kodak No. 1980) and *β*-pinene (Light & Co., unnumbered) gave the sorptograms — n_D^{25} plotted against the 5 µl fraction number — 1.4704_{US}, 1.4703_{1-6}, 1.4710_7, and 1.4764_{US}, 1.4762_{1-5}, 1.4763_6, 1.4771, respectively *.

Ten extractions with 90 % methanol, the methanol portions being 10 % of the terpene volume, removed the "peaks", and after washing with water and drying (Na_2SO_4) index-homogeneous sorptograms were obtained, $1.4703_{US,\,1-7}$ and $1.4762_{US,1-7}$. There was no change after vacuum distillation through an effective column. Measured at different temperatures the refractive indices were: $n_D^{15.8}$ 1.4742 and 1.4805, n_D^{20} 1.4725 and 1.4788, and n_D^{30} 1.4656 and 1.4718, respectively.

Samples of dipentene, both commercial and those prepared in the laboratory, gave no indication of index homogeneity upon sorption analysis even after prolonged fractional distillation. A further sample of dipentene was then prepared by mixing *d*- and *l*-limonene. The *l*-limonene was prepared in the following way. Ten cones of *Pinus alba* Mill. which, according to Brühl [4], contains *l*-limonene were extracted with ether and the turpentine collected

* In order to simplify the printing of the sorptograms and to avoid printed figures the n_D^{25} values of the sorption are given with the fraction number as index. The value of the unsorbed sample is given with US as index.

(~ 4 ml) was distilled through a micro column under vacuum. 4 fractions were obtained giving the sorptograms:

I	1.3898_{US}	1.4658_1	1.4350_2	1.3568_3	1.3503_4	1.3503_5	1.3530_6	1.3560_7
II	1.4630_{US}	1.4632_1	1.4640_2	1.4656_3	1.4682_{4-5}	1.4700_6	1.4702_7	1.4703_8
III	1.4689_{US}	1.4632_1	1.4652_2	1.4688_3	1.4702_4	1.4703_{5-7}		
IV	1.4722_{US}	1.4695_1	1.4702_2	1.4709_{3-5}	1.4712_6	1.4756_7		

120 μl of fraction III were displaced with ethanol through a 50 cm column (i. d. 1.5 mm) packed with activated gel and 10 μl fractions were collected. Refractive indices were determined on 5 μl aliquots of the fractions giving the enlarged sorptogram with an *l*-limonene plateau $n_D^{25} = 1.4703$ (Fig. 1). The residual 5 μl samples of *l*-limonene were mixed with the same amount of *d*-limonene and analysed by sorption in the usual way, giving the sorptogram for dipentene $1.4703_{US, 1-7}$. — The optical rotation of the sample of *l*-limonene was determined on a micro scale using a 5 cm tube, volume 0.33 ml. 10 μl index-homogeneous sample were dissolved in 0.4 ml ethanol, giving $[\alpha]_D^{25} = -100$, assuming the density to be 0.80.

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Isomerization of β-Pinene

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β-Pinene can be converted quantitatively to α-pinene by palladium in the presence of hydrogen. Using an analytical micro sorption method it can be shown that the isomerization is almost always followed by a very slight hydrogenation and a small second isomerization. These secondary reactions occur to about the same extent on treatment of index-homogeneous Δ^3-carene, *d*-limonene, α-pinene and terpinolene with the catalyst.

The isomerizations can be performed on a half micro scale and, in combination with micro sorption analysis, the method can be used to detect and estimate β-pinene in terpene mixtures.

In the field of terpenes, isomerizations are easily caused by the action of suitable agents. Most of these conversions are such that a complex mixture of products is formed. In the case of liquid terpene hydrocarbons it is very difficult to isolate pure compounds from these mixtures, and especially difficult to determine the quantities of the various compounds formed by isomerization. This can be clearly understood from, *e. g.*, the small boiling range of the terpenes, their ability to undergo further isomerizations during chemical isolation and their lack of evident physical identification properties; *cf.*, however, the work by O'Connor and Goldblatt [1].

Richter and Wolff [2] have reported the isomerization of β-pinene to α-pinene by palladium-black to be quantitative, but their experimental proofs for such a reaction are not fully convincing. Comparing the physical constants given by Richter and Wolff — the calculated n_D^{25} values are given in Table 1 — with those of terpenes purified to index homogeneity, see Widmark[3,4], one can assume most of their isomerized samples to be partly hydrogenated. It is not possible to calculate the extent of hydrogenation from their unanalysed refractive indices, as pinane is known to exist in *cis*- and *trans*-forms and as will be shown in this paper, the isomerization is followed by a second isomerization which forms products of higher refractive indices.

Most of the isomerizations by Richter and Wolff were carried out in ether solution but they have demonstrated the reaction to proceed (at a reduced rate) even without any solvent. Their reactions were performed on a macro scale using about 10 % palladium-black and the isomerization products were

Table 1. Calculated n_D^{25} values from Richter and Wolff's[2] isomerizations.

Starting material	n_D^{25}	After isomerization		n_D^{25}
d-α-Pinene	1.4629 [a]	6 h	8 % Pd	1.4628
d-α-Pinene	1.4631	6 h	7.5 » Pd	1.4615
l-α-Pinene	1.4659	6 h	17.5 » Pd	1.4638
β-Pinene	1.4767 [b]	7 h	8 » Pd	1.4624
β-Pinene	»	6 h	— » Pd	1.4622
β-Pinene	»	1 h [c]	— » Pd	1.4606
β-Pinene	»	—[c]	— » Pd	1.4601

a) Index-homogeneous α-pinene = 1.4632, *cf.* Widmark [3]
b) » β-pinene = 1.4763, *cf.* Widmark [4]
c) by shaking.

distilled at atmospheric pressure. They found the presence of hydrogen necessary — fully in accordance with the findings of the present author — and they introduced the hydrogen by bubbling or in some cases by shaking. Moreover, they found that when the catalyst was saturated first with hydrogen the isomerization could proceed in a nitrogen atmosphere; *cf.*, however, Fig. 1, curve c. As seen in Table 1, there was a more marked hydrogenation in their isomerizations when shaking was employed.

In this investigation it was found most convenient — from an analytical point of view — to isomerize the undiluted terpenes (half micro scale) by shaking and to use about half a percent of palladium on barium carbonate as catalyst. This catalyst can be prepared under more controlled conditions than palladium-black. To minimize hydrogenation, the isomerizations were carried out in small ground-glass stoppered test tubes with a maximum free space of 2 ml of hydrogen gas. Isomerization produced by bubbling hydrogen at a slow rate over the catalyst was found to give irregular results. After the isomerization the terpenes were analysed for purity by a micro sorption method devised by Blohm [5]. From these analyses it is clearly demonstrated that the catalytic isomerization of β-pinene to α-pinene is a quantitative reaction, followed, however, by a small second isomerization.

The activity and the properties of the catalyst were investigated by determination of the time necessary for full conversion of β-pinene and by sorption analysis of the products formed.

ACTION OF THE CATALYST ON TERPENES

α-Pinene. Small samples of α-pinene, purified to index-homogeneity [3], were shaken with controlled (see experimental part) Pd-$BaCO_3$-catalyst under different conditions. On sorption analysis, sorptograms — n_D^{25} plotted against the 5 μl fraction numbers — were obtained, which showed mostly a slight upward slope in the beginning and a low peak at the end. It has not yet been found possible to calculate the amount of hydrogenation and isomerization products by comparison with sorptograms of mixtures of known composition

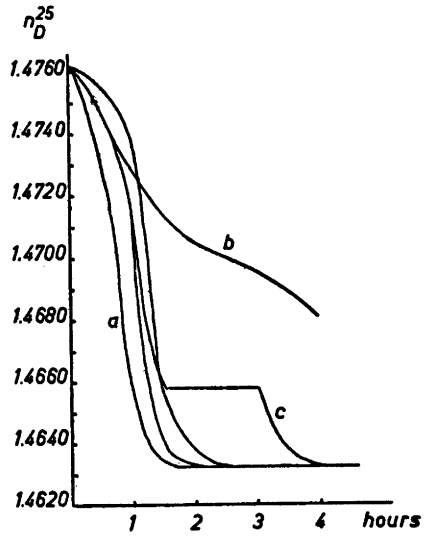

Fig. 1. Rate of isomerization. a) Normal rates using 50 to 200 mg Pd-$BaCO_3$ or 10—200 mg Pd-black. b) Incomplete isomerization using 2 mg Pd-black. c) Isomerization when the hydrogen was temporarily omitted.

as the peak constituent is unknown and the slope product, very probably pinane, has not yet been prepared in a state of index homogeneity. However, from sorptograms of mixtures of other index-homogeneous terpenes (*cf.* Widmark and Blohm [6]) one can assume the formation of by-products to be not more than a few percent each. Until a more accurate method is found the result of the isomerizations will be deduced from the figure of the sorptograms.

On shaking α-pinene with catalyst for one, four and twelve hours, there was a slight increase in both slopes and peaks. However, the variations caused by different samples of catalyst — prepared in exactly the same way — were almost of the same size.

Similar results were also obtained when the reaction was carried out in ether solution, when palladium-black was used, when the hydrogen was bubbled through the material, or when a freshly oxidized sample with a peak was employed.

β-Pinene. The time for full conversion of β-pinene to α-pinene varied mostly from half an hour to four hours depending on the sample of catalyst employed. Only those samples giving conversion within two hours were considered sufficiently active for use in this investigation. There was some dependency on the amount of catalyst used, but as seen from Fig. 1 very small amounts gave a sufficiently high rate of isomerization. 50 mg 5 % Pd-$BaCO_3$ was found convenient to use for one millilitre samples. Calculated on the amounts of palladium, Pd-$BaCO_3$ gave a more rapid isomerization than Pd-black when very small amounts of catalyst were used. The presence of hydrogen was found to be essential for isomerization and when a tube was not properly filled with hydrogen no conversion took place (curve c, Fig. 1).

On sorption analysis after isomerization, sorptograms were obtained similar to those from α-pinene after treatment with the catalyst. One can therefore assume the second isomerization product to be formed from α-pinene.

Δ³-Carene, d-limonene and terpinolene. In a previous communication [3] this author has studied the occurrence of β-pinene in Swedish sulfate turpentine. The content of β-pinene was calculated by comparing the sorptograms of isomerized and unisomerized samples, and by determining the amounts of β-pinene necessary to bring the sorptograms up to their original shapes. This analytical method was found useful, but upon isomerization of the last turpentine fractions unusual sorptograms were obtained which were explained by the presence of terpenes other than Δ³-carene and α- and β-pinene in the turpentine mixture.

It was therefore of interest to determine the action of the catalyst on other index-homogeneous terpenes, both alone and mixed with β-pinene. It was found that the changes in the sorptograms of Δ³-carene, *d*-limonene and terpinolene were almost the same as, or less than, in the case of α-pinene.

On isomerization of two component mixtures of these pure terpenes, with β-pinene as the minor constituent, sorptograms were obtained which resemble — with the exception given above — the ones containing the corresponding amount of α-pinene. On isomerization of a mixture of 17 % β-pinene, 10 % *d*-limonene, and 73 % Δ³-carene, a sorptogram was obtained which differed considerably from that of a mixture of 15 % α-pinene, 10 % *d*-limonene, and 75 % Δ³-carene. This observation was of interest as the last fractions of sulfate turpentine gave sorptograms, before and after isomerization, which resembled the corresponding ones from the first mixture.

Examination of the use of the catalytic isomerization method as an analytical tool to detect and calculate roughly the amount of β-pinene in turpentine mixtures, reveals that a few percent can be detected in favourable cases, *e. g.* the first fractions in the sulfate turpentine. The limit is set by the slight action of the catalyst on other terpenes. With small contents of β-pinene this action has to be controlled with each sample of catalyst. Presence of limonene seems to hamper the interpretation of the sorptograms. However, it is apparent that the isomerization method could be a good analytical aid, in combination with infra-red determinations, when analysing more complex turpentine mixtures.

EXPERIMENTAL PART

Preparation of the catalysts

Palladium on barium carbonate (5 % Pd). Barium carbonate was prepared from 7.5 ml barium chloride solution (33 g $BaCl_2 . 2H_2O$ → 100 ml) and 5.5 ml sodium carbonate (40 g Na_2CO_3 → 200 ml). After decanting and washing several times with water 2.9 ml 1 *N* NaOH were added with vigorous shaking to the wet precipitate followed by 1.66 ml palladium chloride solution (5 g $PdCl_2$ + 12.2 ml conc. HCl → 50 ml), 3.9 ml 1 *N* NaOH and after a minute, 2 drops of 35 % formaldehyde solution. After shaking for 20 minutes the catalyst was washed several times with water, the water being removed by centrifugation. Finally, the catalyst was filtered off using a sintered glass crucible, dried at 80° C, and stored in a desiccator.

Palladium-black. This catalyst was prepared in lots of 0.5 g according to Wieland [7].

Sorption method. A micro sorption apparatus (column 250 × i. d. 1.4 mm), fully conforming to Blohm's instructions, was used. 40.0 μl test samples were displaced with

abs. ethanol through the column and separated into 5 μl fractions. The silica gel (Davison, 922—08—226, through 200 mesh) was activated for 2 hours at 140° and 15 mm Hg. The refractometer, Bellingham & Stanley No. 402330, was calibrated against a standard plate n_D 1.5009. The thermostat was controlled to 25° ± 0.02°. On reading the refractive indices of the 5 μl fractions, a fresh paper ~ 5 × 5 mm was used for each determination.

Isomerization of β-pinene.

Samples of index-homogeneous [4] β-pinene were isomerized using the two catalysts (see above) both by shaking in small ground-glass stoppered test tubes (8 × 50 mm) filled with hydrogen and by bubbling hydrogen at a very slow rate through a narrow capillary over the catalyst. Similar results were obtained in each case, although sometimes the bubbling-method gave irregular results.

One ml samples of β-pinene were isomerized by shaking with different amounts of the two catalysts. The degree of isomerization was determined by reading the refractive indices. There was no marked difference in rate of isomerization using 50 to 200 mg Pd-$BaCO_3$ (5 % Pd) or 10 to 200 mg Pd-black (Fig. 2, curves a). Two mg Pd-black gave only a slow rate of isomerization (curve b) and if the hydrogen was temporarily omitted, there was no isomerization (curve c). The variations caused by using different amounts of catalyst were of the same order as the variations caused by different samples of catalysts. A few samples of catalysts gave, for some unexplained reason, a very slow rate of isomerization even though they had been prepared exactly according to the given instructions.

One isomerization was performed with a catalyst giving a slow rate of isomerization (23 hours) and samples were removed during the isomerization for sorption analyses. These sorptograms * are given below:

3 h	1.4756_{US}	1.4738_1	1.4749_2	1.4754_3	1.4759_4	1.4761_5	1.4762_{6-7}	
5 h	1.4733_{US}	1.4675_1	1.4709_2	1.4768_3	1.4740_4	1.4748_5	1.4752_6	1.4757_7
7 h	1.4710_{US}	1.4642_1	1.4673_2	1.4700_3	1.4718_4	1.4730_5	1.4740_6	1.4749_7
9 h	1.4690_{US}	1.4630_1	1.4647_2	1.4659_3	1.4682_4	1.4707_5	1.4725_6	1.4741_7
12 h	1.4657_{US}	1.4632_{1-3}	1.4640_4	1.4652_5	1.4672_6	1.4700_7		
16 h	1.4642_{US}	1.4632_{1-5}	1.4643_6	1.4654_7				
19 h	1.4633_{US}	1.4632_{1-6}	1.4642_7					
23 h	1.4632_{US}	1.4632_{1-6}	1.4635_7					

Action of the catalysts on α-pinene

The sorptograms of fully isomerized β-pinene resembled closely those obtained from α-pinene after treatment with the catalyst. In nearly every case, a slight slope in the beginning and a small peak at the end of the sorptogram were formed. A few samples of catalysts gave, however, no slope in the beginning of the sorptograms (*cf.* the sorptograms in the preceding paragraph).

Shaking samples (1 ml) of index-homogeneous α-pinene with Pd-$BaCO_3$ (50 mg) for 1, 2, 4, and 12 hours the following sorptograms were obtained:

1 h	1.4632_{US}	1.4629_1	1.4630_2	1.4631_3	1.4632_{4-6}	1.4637_7	
2 h	1.4631_{US}	1.4626_1	1.4629_2	1.4630_3	1.4631_4	1.4632_{5-6}	1.4635_7
4 h	1.4632_{US}	1.4629_1	1.4631_{2-3}	1.4632_{4-6}	1.4635_7		
12 h	1.4632_{US}	1.4629_1	1.4630_2	1.4631_{3-4}	1.4632_{5-6}	1.4640_7	

These sorptograms are similar to the ones using Pd-black, the bubbling method or when the isomerization was performed in ether solution (2 parts of ether). Using freshly oxidized α-pinene or β-pinene as starting material (sorptograms 1.4632_{1-4} 1.4637_5 1.4650_6 1.4686_7 and 1.4762_{1-6} 1.4770_7, respectively) only a slight increase in the peaks was obtained (1.4625_1 1.4629_2 1.4630_3 1.4631_{4-5} 1.4632_6 1.4643_7 and 1.4629_1 1.4630_2 1.4631_3 1.4632_{4-6} 1.4649_7, respectively).

* Sorptogram = n_D^{25} values plotted against 5 μl fraction number. In order to simplify printing of the sorptograms the n_D^{25} values are given with the fraction number as index. The unsorbed value is given US as index.

Action of Pd-$BaCO_3$-catalyst on Δ^3-carene, *d*-limonene and terpinolene

As Pd-$BaCO_3$ was found to be the most convenient catalyst for our purpose the following isomerizations were performed with this catalyst using the shaking method. Shaking samples (1 ml) of index-homogeneous Δ^3-carene, *d*-limonene and terpinolene with 50 mg Pd-$BaCO_3$ for 1, 4 and 12 hours there was an effect similar to the one obtained with α-pinene although the peaks were smaller.

Sorptograms: *Δ^3-Carene*

1 h	1.4700_{US}	1.4698_1	1.4700_{2-7}			
4 h	1.4699_{US}	1.4692_1	1.4698_{2-3}	1.4700_{4-6}	1.4703_7	
12 h	1.4699_{US}	1.4693_1	1.4697_{2-4}	1.4698_5	1.4700_{6-7}	

d-Limonene

1 h	1.4703_{US}	1.4701_1	1.4703_{2-6}	1.4704_7		
4 h	1.4703_{US}	1.4700_1	1.4701_2	1.4703_{3-7}		
12 h	1.4701_{US}	1.4693_1	1.4701_2	1.4702_{3-4}	1.4703_{5-6}	1.4704_7

Terpinolene

1 h	1.4863_{US}	1.4863_{1-6}	1.4869_7		
4 h	1.4862_{US}	1.4859_1	1.4862_{2-6}	1.4864_7	
12 h	1.4862_{US}	1.4862_{1-6}	1.4865_7		

Isomerization of β-pinene mixtures

The index-homogeneous terpenes given above were mixed with small amounts of β-pinene and isomerized. The sorptograms before and after isomerization are given below:

Δ^3-Carene + 5 % β-pinene. 1.4701_{US} 1.4700_1 1.4701_{2-5} 1.4702_6 1.4703_7;
after isomerization 1.4700_{US} 1.4698_1 1.4701_{2-6} 1.4703_7.

d-Limonene + 5 % β-pinene. 1.4708_{US} 1.4710_1 1.4708_{2-3} 1.4706_4 1.4703_{5-6} 1.4710_7;
after isomerization 1.4701_{US} 1.4679_1 1.4694_2 1.4701_3 1.4702_4 1.4703_{5-6} 1.4704_7.

d-Limonene + 10 % β-pinene. 1.4710_{US} 1.4718_1 1.4710_2 1.4707_3 1.4705_4 1.4703_{5-6} 1.4705_7;
after isomerization 1.4700_{US} 1.4669_1 1.4691_2 1.4701_3 1.4703_{4-6} 1.4704_7.

Terpinolene + 2 % β-pinene. 1.4861_{US} 1.4854_1 1.4860_2 1.4861_{3-5} 1.4862_{6-7};
after isomerization 1.4861_{US} 1.4852_1 1.4859_2 1.4861_{3-4} 1.4862_{5-6} 1.4867_7.

Terpinolene + 5 % β-pinene. 1.4859_{US} 1.4843_1 1.4854_2 1.4859_3 1.4860_{4-6} 1.4862_7;
after isomerization 1.4849_{US} 1.4817_1 1.4850_2 1.4853_3 1.4856_4 1.4858_5 1.4780_6 1.3970_7.

Terpinolene + 10 % β-pinene. 1.4853_{US} 1.4822_1 1.4842_2 1.4850_3 1.4854_4 1.4858_5 1.4859_6 1.4860_7;
after isomerization 1.4834_{US} 1.4757_1 1.4832_2 1.4846_3 1.4851_4 1.4853_5 1.4856_6 1.4030_7.

Δ^3-Carene + 10 % limonene + 17 % β-pinene (slightly oxidized). 1.4719_{US} 1.4708_1 1.4705_2 1.7403_3 1.4708_{4-6} 1.4718_7;
after isomerization 1.4728_{US} 1.4703_1 1.4708_2 1.4709_3 1.4710_{4-6} 1.4754_7.

Δ^3-Carene + 10 % d-limonene + 15 % α-pinene. 1.4682_{US} 1.4667_1 1.4682_2 1.4690_3 1.4691_4 1.4693_5 1.4697_6 1.4701_7.

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Infrared Spectra of Alkylsilanes I

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The infrared spectra of 26 alkylsilanes and 2 phenylsilanes have been recorded between 2—15 μ. The wavelength shifts of $=SiH$, $=SiH_2$ and $-SiH_3$ groups in the range 4.6—4.8 μ are reported as well as key bands of alkylsilanes containing $=SiH_2$, $-SiH_3$, $=Si(CH_3)_2$ and $-Si(CH_3)_3$ in the range 10—15 μ.

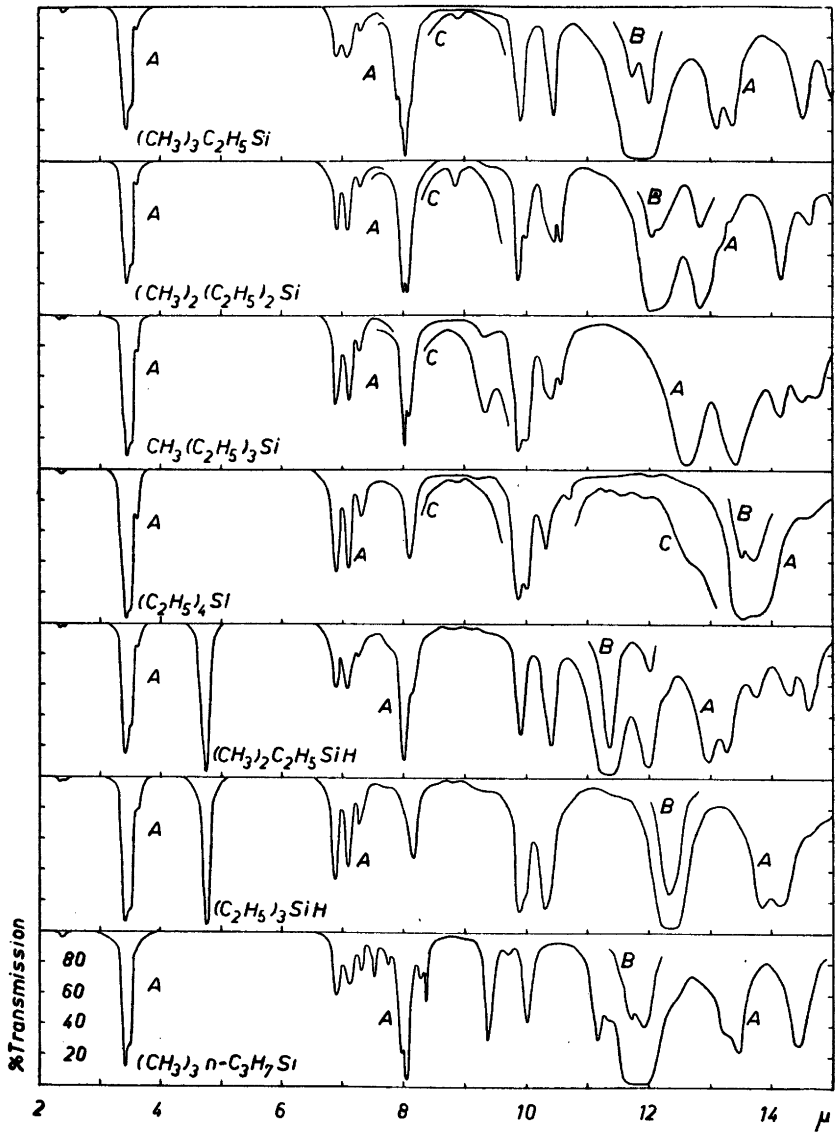
Data on infrared absorption intensities of organic silicon compounds are scarce in the literature. A comparison of spectra of homologues of *n*-alkylsilanes revealed a qualitative relationship between the length of *n*-alkyl-groups and the intensity of some absorption bands. In a subsequent paper numerical data on the intensities of five absorption bands between 6.8 and 8.5 μ will be given.

All the silicon hydrides strongly absorbed at 4.6—4.75 μ. It was found that the absorption wavelength was dependent on the number of hydrogen atoms bonded to the silicon atom in alkylsilanes. Thus all monoalkylsilanes (—SiH_3) absorbed at 4.62 μ, the dialkylsilanes (= SiH_2) at 4.68—4.69 μ and the trialkylsilanes (≡ SiH) at 4.74 μ. Diphenylsilane absorbed at 4.64 μ and triphenylsilane at 4.68 μ.

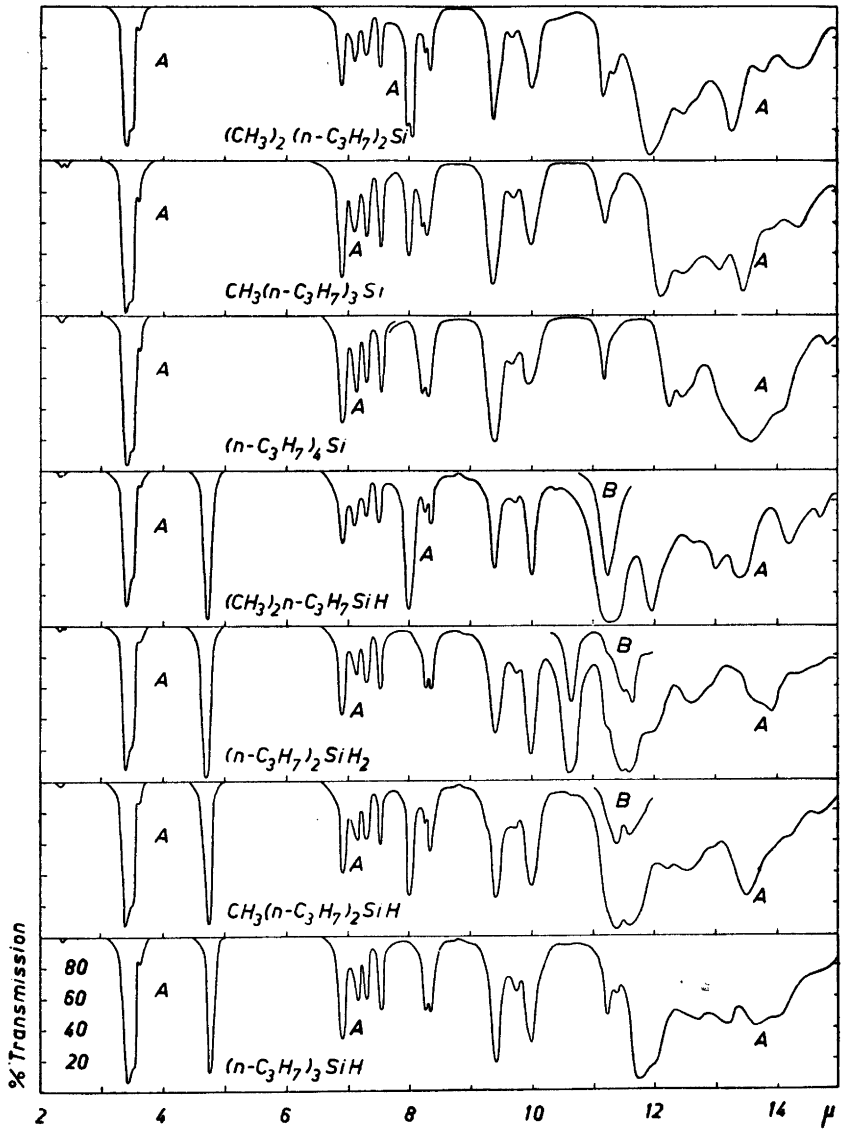
Of the three alkylsilane absorption bands between 6.8 and 7.3 μ (see the figures on pp. 948—951) two are also found in the spectra of paraffins. The C—H bending vibrations in methyl (asymmetric) and methylene groups appear at 6.85—6.86 μ and the symmetric C—H bending vibrations of the methyl groups at 7.27 μ. Near 7.1 μ alkyl silanes give absorption which is not found for corresponding alkanes. Alkylsilanes containing propyl or higher *n*-alkyl groups absorb at about 7.5 μ. For butyl or longer chains there are some weak absorption bands between 7.5—8.0 μ, in which range CH_2 wagging and twisting modes appear in the alkane spectra.

In the range 7.8—8.6 μ the alkylsilanes absorbed in the way described by Kay and Tannenbaum [1] and Harvey *et al* [2].

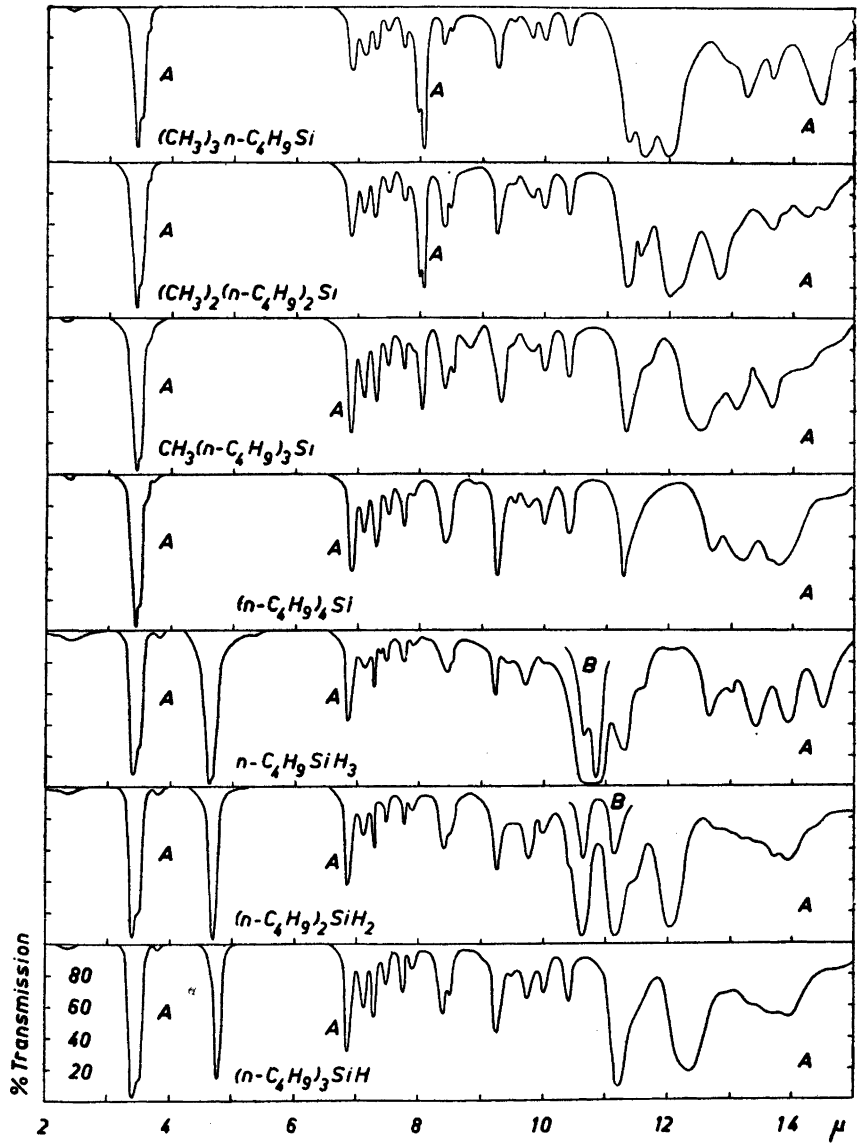
The range 10—15 μ shows absorption bands which are useful for the correlation of chemical structures of alkylsilanes. All the monoalkylsilanes investigated showed strong absorption in 10 % solution in carbon disulfide at 10.6—11.0 μ which on dilution to 1 % solution resolved into two bands at 10.6—



10.7 μ (medium) and 10.85 μ (very strong). The $-SiH_3$ group gave a characteristic triplet of bands of moderate strength at 13.4—13.5, 14.0 and 14.4—14.5 μ. The dialkylsilanes, *i. e.*, compounds containing the $=SiH_2$ group, all gave a strong band in 10 % solution, medium in 1 % solution, at 10.6—10.7 μ. However, the very strong band at 10.85 μ for the $-SiH_3$ group was not found here. Weak absorption at 11.2 μ and 14.0 μ was found in the spectra of all dialkylsilanes, but does not seem to be of great value for the correlations.

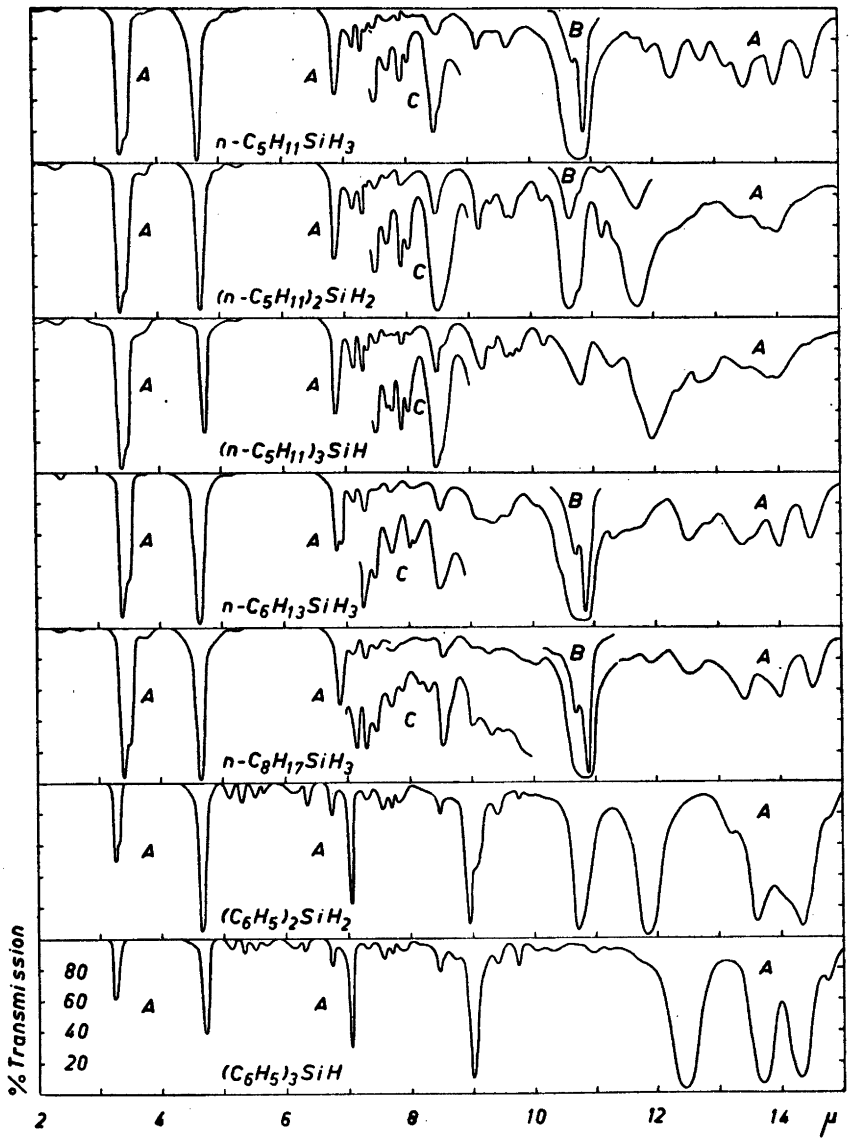


The tetraalkylsilanes containing the trimethylsilyl group showed strong absorption at 11.6—12.1 μ in 10 % solutions in carbon disulfide. In 1 % solution the absorption resolved into two bands of about equal strength at 11.6—11.7 μ and 12.0 μ. Wright and Hunter [3] reported the Si—C stretching mode to appear at 11.63 μ in tetramethylsilane and at 11.88 μ in terminal groups —O—Si$(CH_3)_3$ of linear methylpolysiloxanes. Further the trimethylsilyl group gave a less prominent band at 14.4—14.5 μ. The alkylsilanes containing



the group $=Si(CH_3)_2$ also show strong absorption at 12.0 μ in both 10 % and 1 % solutions. This type of compounds can be distinguished from alkyltrimethylsilanes through the absence of the 11.7 μ band.

All the bands in the range 10—15 μ described here are, if not otherwise stated, characteristic for the configurations of the investigated substances and should be suitable for identification purposes.



EXPERIMENTAL

The spectra were recorded with a Beckman spectrophotometer model IR-2, equipped with a rock salt prism, and a Brown Electronik pen recorder. Calibration of the wavelength scale was carried out against the values given by Oetjen *et al.*[4] for CO_2: 4.225, 14.97 μ and H_2O: 6.903 μ. The cell thickness was 0.10 mm. All spectra were measured in about 10 % solutions in the wave length range 2—15 μ. In some cases spectra of pure substances or of about 1 % solutions were recorded. Carbon tetrachloride was used as the

solvent between 2 and 8 μ and carbon disulfide in the range 7.6—15 μ. In the figures on pp. 948—951 the concentrations are given as follows: A *ca.* 10 %; B *ca.* 1 %; C = pure substance.

All molecular refractivities were calculated in the manner previously described [5]. The bond refractivity of the silicon chlorine bond, 7.20, given by Warrick [6] was used.

Preparative part

The substances containing Si-H bonds were prepared by reduction of alkylethoxysilanes [5] or of chlorosilanes with lithium aluminium hydride. The chlorosilanes were prepared either from silicon tetrachloride and alkylmagnesium chloride or from disiloxanes according to the methods of Flood [7] or Voronkov *et al.*[8]

The tetraalkylsilanes were prepared from alkylchlorosilanes and alkylmagnesium chloride or bromide or purified from material available in this laboratory. Dimethyldi-*n*-propylsilane and methyltri-*n*-propylsilane were identical to the samples previously reported [9]. The methyl-*n*-butylsilanes were samples from the work of Larsson and van der Gilse van der Pals [10]. The refractive indices of the used samples were remeasured.

Trimethylethylsilane and *trimethyl-*n*-propylsilane* were prepared from trimethylchlorosilane and the appropriate Grignard reagent according to method II of Whitmore *et al*[11]. (These authors used another method for the preparation of the substances described here).

The preparation of trimethyl-*n*-propylsilane may be described as an example for both syntheses.

n-Propylmagnesium chloride was prepared from 7.2 g (0.3 g atom) of magnesium, 23.7 g (0.3 mole) of *n*-propyl chloride, and 40 ml of anhydrous ether in a 500 ml apparatus. To the solution was added 11 g (0.1 mole) of trimethylchlorosilane dissolved in 15 ml anhydrous ether during 20 minutes and the reaction mixture was boiled for one hour. After hydrolysis with 5 *N* hydrochloric acid, distillation of the product gave 7.5 g (65 %) of trimethyl-*n*-propylsilane boiling at 88° (1 atm). n_D^{20} 1.3932.

Ethyl bromide was used in the preparation of trimethylethylsilane. The yield was 60 %. B. p. 62°. n_D^{20} 1.3832.

Methyltriethylsilane was prepared from triethylchlorosilane and Grignard reagent according to Whitmore *et al.*[11] Methylmagnesium chloride was used instead of the bromide. The yield of methyltriethylsilane was 56 %. B. p. 126—127° (1 atm). n_D^{20} 1.4165.

Dimethylethylchlorosilane was prepared by a Flood reaction. 1,1,3,3-tetramethyl-1,3-diethyldisiloxane (127 g) was shaken with 100 ml of concentrated sulphuric acid. To the solution was added 75 g of sodium chloride and 75 g of ammonium chloride in portions. The upper layer was collected and distilled, giving 105 g of dimethylethylchlorosilane boiling at 87—92° (1 atm).

Dimethylethylsilane. To a slurry of 8.0 g (0.21 mole) of lithium aluminium hydride in 15 ml of anhydrous dibutyl ether was added 92 g (0.75 mole) of dimethylethylchlorosilane during half an hour. The reaction was spontaneous and accompanied by the evolution of heat. When about half of the chlorosilane had been added, slight external heating was applied and the dimethylethylsilane was removed continously through a short Vigreux column. The yield of dimethylethylsilane was 63.6 g (95 %) boiling at 44—46°. (Found: Si 32.0. Calc. for $C_4H_{12}Si$: Si 31.8). d^{20} 0.6679; n_D^{20} 1.3789; MR_D found 30.52, calc. 30.46.

*Dimethyl-*n*-propylchlorosilane* was prepared from 56 g of 1,1,3,3-tetramethyl-1,3-di-*n*-propyldisiloxane (see below), 40 ml of concentrated sulphuric acid and 40 g of ammonium chloride. Distillation yielded 56 g of dimethyldi-*n*-propylchlorosilane boiling at 113—115°. (Found: Si 20.6 Calc. for $C_5H_{13}ClSi$: Si 20.5). d^{20} 0.8753; n_D^{20} 1.4133; MR_D found 38.97, calc. 39.20.

*1,1,3,3-tetramethyl-1,3-di-*n*-propyldisiloxane* was prepared from dimethyl-*n*-propylethoxysilane. (Of course it is more convenient to use the ethoxysilane directly for the preparation of the chloride, without isolating the disiloxane).

To 36 g (0.25 mole) of ice-cooled dimethyl-*n*-propylethoxysilane was added slowly 50 ml of concentrated sulphuric acid. After standing for 14 hours the mixture was poured out on ice. Two layers were formed and separated. The diluted sulphuric acid solution was extracted with ether. The ether solution of all organic material was washed with

water, 25 ml of a 5 % sodium carbonate solution, water again and dried with calcium chloride. Distillation yielded 25.2 g of tetramethyldi-*n*-propyldisiloxane boiling at 66° (11 mm). (Found: Si 26.6. Calc. for $C_{10}H_{26}OSi_2$: Si 26.5). d^{20} 0.8027; n_D^{20} 1.4098; MR_D found 67.41, calc. 67.46.

*Methyldi-*n*-propylchlorosilane* was prepared from methyldi-*n*-propylethoxysilane analogous to dimethyl-*n*-propylchlorosilane, but the disiloxane was not isolated. (See below). Methyldi-*n*-propylchlorosilane boiled at 161° (1 atm). (Found: Si 17.0. Calc. for $C_{17}H_{17}ClSi$: Si 17.0). d^{20} 0.8784; n_D^{20} 1.4300; MR_D found 48.44, calc. 48.46.

*1,3-Dimethyl-1,1,3,3-tetra-*n*-propyldisiloxane* was prepared from methyldi-*n*-propylethoxysilane in a separate experiment, as described for tetramethyldi-*n*-propyldisiloxane. B. p. 115—116° (12 mm). (Found: Si 20.5. Calc. for $C_{14}H_{34}OSi_2$: Si 20.5). d^{20} 0.8217; n_D^{20} 1.4290; MR_D found 86.15, calc. 85.99.

The following four alkylsilanes were prepared by reduction of corresponding alkylchlorosilanes with lithium aluminium hydride in diethyl ether:

Silane:	B. p. °C	d^{20}	n_D^{20}	MR_D found	MR_D calc.
Dimethyl-*n*-propyl-	73—74	0.6976	1.3927	34.96	35.21
Methyldi-*n*-propyl-	127	0.7327	1.4140	44.43	44.48
Triethyl-	107	0.7313	1.4124	39.60	39.40
n-Butyl-	54	0.6772	1.3926	31.07	31.06

n-*Octylsilane* was also prepared by reduction of the *n*-octyltrichlorosilane by a modified method, where the chlorosilane was never isolated. *n*-Octylmagnesium bromide was prepared from 49 g (0.25 mole) of *n*-octyl bromide and 6.1 g (0.25 g atom) of magnesium in 50 ml of anhydrous ether. The Grignard reagent was filtered and added to a stirred solution of silicon tetrachloride, 43 g (0.25 mole) in 75 ml of ether, during 40 minutes. After rapid filtration through glass wool the solvent and the unreacted silicon tetrachloride were removed by distillation in order to prevent the formation of SiH_4 in the following reduction. Anhydrous ether (*ca.* 75 ml) was again added and the solution was introduced into a stirred slurry of 9 g (0.25 mole) of lithium aluminium hydride in 50 ml of anhydrous ether.

Direct distillation at 20 mm yielded 19 g (50 %) of *n*-octylsilane boiling at 59—63°. After redistillation the following data were found. B. p. 61° (20 mm). (Found: Si 19.4. Calc. for $C_8H_{20}Si$: Si 19.5). d^{20} 0.7457; n_D^{20} 1.4253; MR_D found 49.52, calc. 49.61.

The distillation residue was hydrolysed with diluted hydrochloric acid. No di-*n*-octylsilane could be isolated.

Triphenylsilane. Triphenylchlorosilane was prepared by adding phenyllithium (0.31 mole as a 0.94 *N* ether solution) to silicon tetrachloride (0.1 mole) dissolved in ether (100 ml). The precipitate was removed and the ether solution was added to 1.2 g (0.032 mole) of lithium aluminium hydride in ether (100 ml). After completion of the reduction the reaction mixture was rapidly filtered and about 400 ml of ether was distilled off. Excess of lithium aluminium hydride was decomposed by adding the solution to 50 ml of 5 *N* hydrochloric acid. The ether layer was dried with calcium chloride and distilled, yielding 11 g of triphenylsilane, boiling at 148—151° at 1 mm. After one hour the substance solidified. M. p. 35°.

The refractive indices of the following compounds will be given: dimethyldiethylsilane 1.4014, tetraethylsilane 1.4270, tetra-*n*-propylsilane 1.4386, tetra-*n*-butylsilane 1.4468.

I should like to express my appreciation to the director of this institute, Professor Erik Larsson, for his interest in this work.

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Über die Umkehrbarkeit der Reaktion zwischen Oxoverbindungen und Formaldehyd. Auf- und Abbau der β-Oxoalkohole. Trans-Oxymethylierungs- und Krypto-Formaldehyd-Reaktionen

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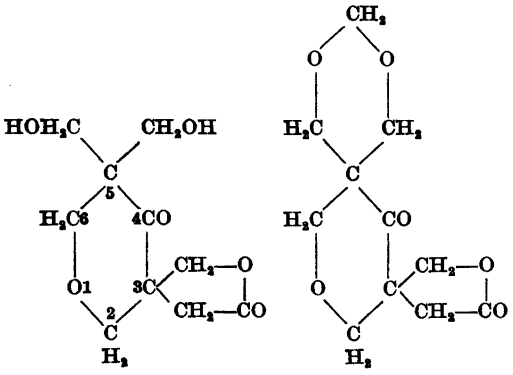
It is shown that *β-oxoalcohol systems* in acid, alkaline and neutral solution are able not only to split off hydroxymethyl groups in the form of formaldehyde, but moreover to transfer them to other suitable compounds. If the hydroxymethyl group is transferred unchanged from the donor to the acceptor system, this type of reaction is called "*trans-hydroxymethylation*" in analogy to other phenomena of biological group transfers, such as trans-amination, trans-phosphorylation *etc.* — In contrast to the amino or phosphate groups, the hydroxymethyl group will after having been transferred to the acceptor system often suffer a change by secondary processes, and therefore, appear in the acceptor system in a modified form, *e. g.* as a methyl, methylene, aldehyde or carboxyl group. The totality of the appearances caused by the action of formaldehyde, released from a real carbon-carbon bond or other bonds, and such reacting in a hidden manner, is collected under the designation of "crypto-formaldehyde-reactions", the consequences of which for chemical and biochemical investigations are discussed on an experimental basis.

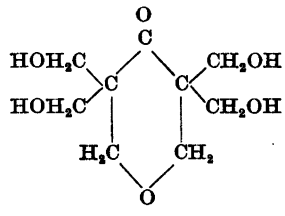
Wie aus dem historischen Versuch Butlerows [1] und späteren Arbeiten anderer namhafter Forscher bekannt ist, vermag sich Formaldehyd in schwach alkalischem Medium über die Zwischenstufe des Glykolaldehyds [2] mit sich selbst zu Zuckern zu kondensieren. Tollens [3] beobachtete, dass unter gleichen Milieu-Bedingungen auch andere Aldehyde und Ketone sich mit überschüssigem Formaldehyd unter intermediärer Bildung von *β-Oxoalkoholen* zu mehrwertigen Alkoholen kondensieren lassen. Aus früher dargelegten Gründen [4] kommt jedoch der alkalische Methylolaufbau zur Darstellung mehrwertiger *β-Oxoalkohole* praktisch nicht in Betracht. Erst die Erkenntnis, dass sich Carbonylverbindungen ganz allgemein in Eisessig-Schwefelsäure erschöpfend oxymethylieren lassen, und dass bei dieser Reaktion — auch bei Anwendung

eines grossen Überschusses von Formaldehyd — bei *Ketonen* die Carbonylgruppe nicht reduziert wird [4–7], ermöglichte die mühelose Darstellung und ein näheres Studium dieser interessanten Verbindungsklasse. — Versetzt man eine wässrige Lösung des durch erschöpfende Oxymethylierung nach dem neuen Verfahren dargestellten *3,5,5-Trimethylol-dihydro-desoxy-patulinsäurelactons*(I) bei Zimmertemperatur mit einer kalt gesättigten *Barytlösung*, erhält man in einer nach wenigen Minuten entnommenen, mit Salzsäure angesäuerten Probe mit salzsaurem *2,4-Dinitrophenylhydrazin* einen kräftigen gelben Niederschlag, mit *fuchsinschwefliger Säure* eine stark positive Reaktion auf *Formaldehyd*. Bei sofortiger Destillation der Mischung lassen sich im Destillat reichliche Mengen Formaldehyd nachweisen. Überlässt man dagegen die Mischung sich selbst, erhält man mit den genannten beiden Reagenzien am nächsten Tage eine schwächere, nach mehrtägigem Stehen überhaupt keine Reaktion. — Tilitschenko [8] konnte für die alkalische Kondensation des *Methyl-äthyl-ketons* mit Formaldehyd zeigen, dass die Cannizzaro-Reaktion durch das bei der Reaktion entstehende *3,3-Bis-oxymethyl-butanon*-(2) beschleunigt wird, und er hat diese beschleunigende Wirkung auf "naszierenden" Formaldehyd zurückgeführt, der bei der rückläufigen Spaltung des *3,3-Bis-oxymethyl-butanon-(2)* entsteht. Auf die Umkehrbarkeit der Reaktion zwischen *Ketoparaconsäureester* und Formaldehyd haben in neuerer Zeit Gault und Fischhof [9] hingewiesen. Die Abspaltung von Formaldehyd aus triterpenoiden Naturstoffen mit einer β-Ketonalkohol-Gruppierung in alkalischem und saurem Milieu haben Barton und de Mayo [10] beobachtet. — Aus unserem Versuch folgt nicht allein die prinzipielle Umkehrbarkeit der alkalischen Oxymethylierungs-Reaktion, sondern er gibt darüber hinaus einen gewissen Aufschluss über das Schicksal der bei der Spaltung primär entstehenden Fragmente, nämlich des *Formaldehyds* und des nach dessen Abspaltung hinterbleibenden "*Carbonylrumpfes*". Da man — wie beim Methylolaufbau — auch beim alkalischen *Methylolabbau* mit einer Cannizzaro-Reaktion rechnen muss, haben wir bezüglich unseres Versuches aus dem Verschwinden der Formaldehyd- und der Ketocarbonyl-Reaktion geschlossen, dass das Carbonyl-Fragment durch eine gekreuzte Cannizzaro-Reaktion mit dem Formaldehyd zum *Alkohol* reduziert wird, und dass der dann noch verbleibende überschüssige Formaldehyd sich zum grössten Teil

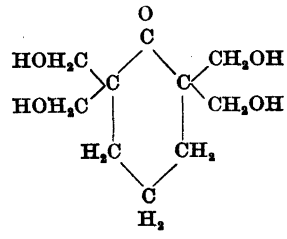


I

II



III



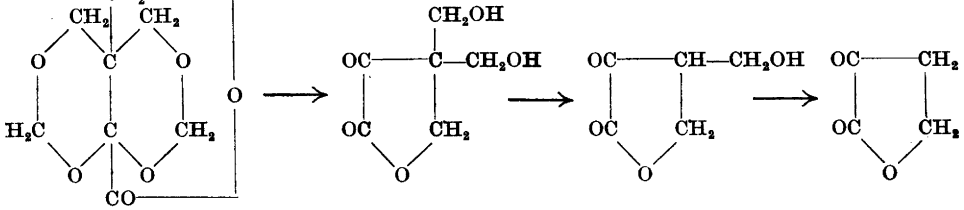
IV

ohne weiteres disproportioniert. Hierbei muss man bedenken, dass der stufenweise Methylol*aufbau* wie der Methylol*abbau* durch die gekoppelte Cannizzaro-Reaktion nur bedingt reversible Vorgänge darstellen, da Auf- und Abbau in dem Augenblick beendet sind, wo die Oxoverbindung zum Alkohol reduziert wird und dadurch aus dem Gleichgewicht ausscheidet.

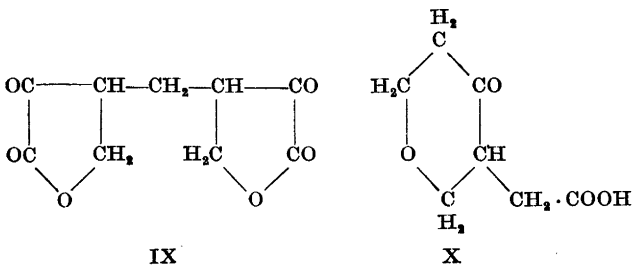
Wir haben uns bemüht, die aus unserem alkalischen Abbau-Versuch hervorgehenden logischen Konsequenzen experimentell zu erfassen, indem wir zunächst andere Verbindungen mit einer *β-Ketonalkohol-Gruppierung* mit Barytlösung prüften. Dabei zeigten die *Anhydro-ennea-heptose (III)* [4] und das *2,2,6,6-Tetramethylol-cyclohexanon*(IV) [5,11] ein völlig gleichartiges Verhalten. Sie stimmen mit dem *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton*(I) auch in der Eigenschaft überein, *Fehlingsche* und *Tollenssche Lösung* zu reduzieren. Der nächste Schritt zur Aufklärung des alkalischen Abbaues besteht in Versuchen, die aus der Spaltung der Per-oxymethylierungsprodukte (I, III und IV) hervorgehenden Fragmente zu identifizieren. Da wir die von uns bisher dargestellten *β-Ketonalkohol-Derivate* dem Methylolaufbau in saurem Milieu verdanken, wobei wir auf die Bedeutung der Essigsäure bereits hingewiesen haben [4], hat es für uns besonders nahe gelegen, den sauren Methylolabbau zu untersuchen. Im folgenden werden unsere Ergebnisse beim sauren, alkalischen und neutralen Methylolabbau behandelt.

I. DER SAURE METHYLOLABBAU

Das *α-Oxo-β,β-dimethylol-butyrolacton (VI)*, das *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton*(I), die *Anhydro-ennea-heptose*(III) und das *2,2,6,6-Tetramethylolcyclohexanon*(IV) verhalten sich gegenüber Mineralsäuren grundsätzlich gleichartig, jedoch scheinen graduelle Unterschiede vorzuliegen. Beim Abbau des *Bismethylenäthers* des *α,α-Dioxy-β,β-dimethylol-butyrolactons (V)*



V VI VII VIII



IX X

durch Salzsäure erhielten wir das *Dilacton*(IX) [6], das nur über die Zwischenstufen VI→VII→VIII durch intermolekulare Kondensation der Fragmente VII und VIII entstanden sein kann. Diese Empfindlichkeit gegen heisse Salzsäure besitzt z. B. das *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton*(I) nicht. Andererseits spalteten die bisher untersuchten Verbindungen (I, III, IV und V) bei der Destillation mit starker Schwefelsäure bzw. Essigsäure-Schwefelsäure-Mischungen bereitwillig Formaldehyd ab, so dass wir erwogen haben, ob sich wertvolle *Zwischenstufen*, die durch Methylol*aufbau* nicht oder nur schwer zugänglich sind, sicherer durch Methylol*abbau* erhalten liessen, nachdem ja die Carbonylfunktion — im Gegensatz zum alkalischen Abbau — in saurem Medium unberührt bleiben sollte.

Wir haben mit dem Lacton I eine ganze Reihe von Abbaustudien durchgeführt und dabei Abbauprodukte von Ketoncharakter erhalten, deren Reinigung und sichere Identifizierung wir zurückstellen mussten, um vorerst nach den Fragmenten des alkalischen Abbaues, der unter milderen Bedingungen verläuft, zu suchen. Beim *β-Acetyl-äthylalkohol* scheint dagegen die Abspaltung von Formaldehyd bevorzugt in saurer bzw. neutraler Lösung zu erfolgen. — Hier kam es uns nur darauf an, zu zeigen, dass die Abspaltung von Methylolgruppen durch Säuren in allen geprüften Fällen möglich war.

II. DER ALKALISCHE METHYLOLABBAU

Durch alkalischen Methylolabbau des *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lactons*(I), wobei der Lactonring geöffnet und die daran beteiligte Methylolgruppe zur Abspaltung freigelegt wird, hofften wir zu der uns bekannten [12] *Dihydro-desoxy-patulinsäure* (X) zu gelangen. Um jedoch gleichzeitig Aufschluss über die Möglichkeit eines partiellen Abbaues zu erhalten, haben wir zunächst die kurzdauernde Einwirkung einer Barytlösung in der Kälte untersucht, indem wir den Abbau schon nach wenigen Minuten durch Ansäuern abstoppten und die Spaltstücke von Aldehyd- bzw. Ketoncharakter mit *2,4-Dinitro-phenylhydrazin* abfingen. Wie erwartet, erhielten wir zunächst das *2,4-Dinitro-phenylhydrazon des Formaldehyds*, dann aber aus dem Filtrat eine Verbindung $C_{15}H_{16}O_8N_4$ vom Schmp. 263°, die das *2,4-Dinitro-phenylhydrazon* einer *Säure* $C_9H_{12}O_5$ von bisher nicht gesicherter Konstitution ist, die sich von dem *Ausgangslacton*(I) $C_{14}H_{14}O_6$ durch Minderbesitz *eines* Moleküles Formaldehyd unterscheidet, das zweifellos bei der Reaktion daraus abgespalten worden ist! Bei einwöchiger Einwirkung einer Barytlösung auf das *Lacton* (I) bei Zimmertemperatur erhielten wir auch nicht die *Dihydro-desoxy-patulinsäure* (X), sondern nach Umsetzung des Reaktionsgemisches mit Benzoylchlorid nach Schotten-Baumann eine carbonylfreie Verbindung vom Schmp. 187°, deren Summenformel $C_{28}H_{26}O_8$ auf das *Tribenzoat* einer von uns noch nicht in freier Form isolierten *Säure* $C_7H_{14}O_5$ stimmt, das aus dem *Lacton* (I) durch Abspaltung von *drei* Molekülen Formaldehyd unter gleichzeitiger Reduktion der Carbonylgruppe und vermutlich anschliessender Ringöffnung und Veresterung durch Benzoylchlorid hervorgegangen ist *. Um die aus diesem Versuch ersichtliche Reduktion durch Formaldehyd zu vermeiden, haben wir

* Über experimentelle Einzelheiten dieser und anderer Abspaltungs-Reaktionen, die noch bearbeitet werden, wird gesondert berichtet.

versucht, diesen bei der Entstehung gleich aus dem Reaktionsgemisch zu eliminieren. Bei kurzdauernder Einwirkung von überschüssigem Tollens-Reagenz, wobei der abgespaltene Formaldehyd zu Formiat oxydiert wird, erhielten wir nach entsprechender Aufarbeitung mit salzsaurem *2,4-Dinitro-phenylhydrazin* ohne weiteres das erwähnte *2,4-Dinitro-phenylhydrazon* vom Schmp. 263°. Zu dem gleichen Resultat führte ein Abbauversuch mit Baryt und Raney-Nickel, das nach Delépine und Horeau [13] die Disproportionierung des Formaldehyds beschleunigen soll. Wir haben uns ausserdem davon überzeugt, dass eine Reihe anderer schwach basisch wirkender Reagenzien, wie Calcium- und Bariumcarbonat in der Kälte oder Natriumacetat in der Siedehitze, aus dem *Lacton*(I) bzw. der *Anhydro-ennea-heptose*(III) Formaldehyd abzuspalten vermögen. Bei der Anwendung von Natron- bzw. Kalilauge wurde in verschiedenen Versuchen neben der Abbau-Reaktion eine gleichzeitige Reduktion der Carbonylgruppe erzielt. Der *Methylenäther* des *3,5,5-Trimethyloldihydro-desoxy-patulinsäure-lactons*(II) wird, wie gezeigt [7], durch Alkalilaugen z. T. in dieser Weise reduziert.

III. DER NEUTRALE METHYLOLABBAU

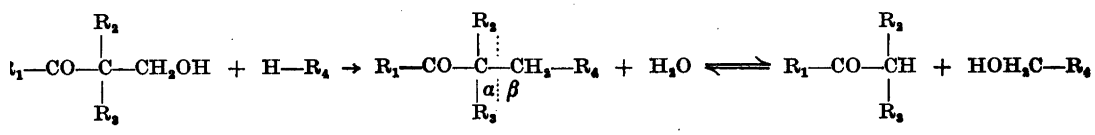
β-Ketonalkohole bzw. deren nicht funktionelle Derivate scheinen allgemein auch in reinem Zustand zur Abspaltung der Methylolgruppen als Formaldehyd bzw. zu seiner intermolekularen Verschiebung befähigt zu sein. So berichten z. B. Bolle und Jullig [14], dass reines *2-Methylol-cyclohexanon* beim Aufbewahren in beträchtlicher Menge in *Cyclohexanon* und *2,2,6,6-Tetramethylol-cyclohexanon* (IV) übergeht. Die Erklärung dieser Autoren, wonach die Abspaltung über das *1-Oxy-methoxy-cyclohexen* erfolgen soll, scheint jedoch durch das Verhalten *per-oxymethylierter Ketone* widerlegt, die trotz mangelnder Enolisierungsmöglichkeit Formaldehyd leicht abspalten. Eine Abspaltung von Formaldehyd erfolgt schon bei der Destillation einer wässrigen Lösung der *Anhydro-ennea-heptose*(III) oder beim Erhitzen dieser Verbindung sowie auch der Verbindungen I und IV über den Schmelzpunkt. Der Abbau des *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lactons*(I) lässt sich sehr gut mit *Dinatriumsulfit* veranschaulichen, das nach Lemme [15] und späteren Autoren mit Formaldehyd quantitativ unter Bildung von *formaldehyd-schwefligsaurem Natrium* und *Natriumhydroxyd* reagiert. Beim Vermischen einer wässrigen Lösung des *Lactons*(I) mit einer genau neutralen Lösung von *Dinatriumsulfit* unter Zusatz von *Thymolphthalein* tritt nach schwachem Erwärmen durch Bildung von Natronlauge zuerst vorübergehend eine schwache Blaufärbung auf, die dann wieder verschwindet, indem das Alkali zur Öffnung des Lactonringes verbraucht wird. Erst wenn die Carboxylgruppe des Lactons neutralisiert ist, kehrt die Blaufärbung durch die Umsetzung des nun abgespaltenen Formaldehyds mit dem Reagenz unter Bildung freier Natronlauge zurück. Zur quantitativen Bestimmung des aus *β-Oxoalkoholsystemen* abspaltbaren Formaldehyds scheint diese Methode wegen eintretender Nebenreaktionen und des dadurch bedingten unscharfen Umschlagspunktes nicht recht geeignet zu sein. — Auch der *β-Acetyl-äthylalkohol* spaltet beim kurzen Erhitzen seiner wässrigen Lösung Formaldehyd ab, der sich mit fuchsinschwefliger Säure nachweisen liess.

IV. TRANS-OXYMETHYLIERUNGS- UND KRYPTO-FORMALDEHYD-REAKTIONEN

Auf Grund der leichten Abspaltbarkeit des Formaldehyds unter verschiedenen Milieubedingungen haben wir uns — ursprünglich aus rein präparativen Erwägungen — gefragt, ob es nicht zweckmässig wäre, beim Methylolabbau den abgespaltenen Formaldehyd "in statu nascendi" fest an ein Molekül anderer Art zu binden und so an der Auslösung unerwünschter Nebenreaktionen zu verhindern. Andererseits unterliegt es ja von vornherein keinem Zweifel und bedarf daher keines experimentellen Beweises, dass Formaldehyd, der mit Leichtigkeit aus dem Verbande des *β-Oxoalkohol-Systems (Methylolgruppen-Donator!)* ausscheidet, in den fester gefügten Verband einer geeigneten anderen *Verbindung (Methylolgruppen-Akzeptor!)* eintreten wird, wenn nur die Milieu- und sonstigen Bedingungen für den Abspaltungs- und Additionsvorgang in gleicher Weise günstig sind. Als Akzeptormolekül kann grundsätzlich jede Verbindung dienen, mit der Formaldehyd zu reagieren vermag. Diese Verhältnisse berühren nicht allein den Synthetiker, sondern können für den Analytiker bei der Untersuchung biologischen Materials weitgehende Konsequenzen haben. Nehmen wir an, dass *β-Oxoalkohol-Verbindungen* integrierende Bestandteile der Zelle wären, besteht — wenn es sich nicht gerade um in Wasser relativ schwerlösliche Verbindungen, wie z. B. die von Barton und de Mayo [10] isolierten, handelt — die Gefahr, dass sie bei dem üblichen Untersuchungsgang (Trennung unter Anwendung von Mineralsäuren, Alkalien, Wasserdampf, Destillation u.s.w.) nicht allein ihrer Erkennung als *genuine* Verbindungen entgehen, sondern darüber hinaus während der Aufarbeitung Methylolgruppen auf andere im Untersuchungsmaterial vorliegende Akzeptormoleküle übertragen und so eine Genuität für Verbindungen vortäuschen, die die Zelle nicht beherbergte und die ihre Entstehung nur einer *"Trans-Oxymethylierung"* verdanken. Im Gegensatz zu anderen bekannten *Gruppenübertragungen* des Stoffwechsels, wie z. B. der *Transaminierung* oder der *Transphosphorylierung*, bei denen die übertragene Gruppe noch im Akzeptormolekül als solche erkennbar ist, *kann* bei einer Trans-Oxymethylierung die übertragene Methylolgruppe im Akzeptormolekül in mannigfacher Weise sekundären Umsetzungen ausgesetzt und daher mehr oder minder verändert sein. Wir bezeichnen daher den gesamten Komplex dieser *primär* durch *Methylolgruppen-Verschiebungen* ausgelösten Erscheinungen als *"Krypto-Formaldehyd-Reaktionen"*, womit das verborgene Wirken des Formaldehyds aus einer echten Kohlenstoff-Kohlenstoff-Bindung oder aus einer anderen Bindung heraus sinnfällig zum Ausdruck gebracht werden soll. Die Bezeichnung besagt nicht etwa, dass der Formaldehyd während des Hinüberwechselns vom Donator- zum Akzeptormolekül analytisch nicht nachweisbar sein soll. Wieweit der Formaldehyd während einer Trans-Oxymethylierungs-Reaktion überhaupt Gelegenheit zu selbständigen Reaktionen hat, hängt ganz von den Gruppenpotentialen des betr. Donator- bzw. Akzeptor-Systems ab im Sinne der von Lipmann [16] für andere Gruppenübertragungen entwickelten Vorstellungen. Andererseits können wir gerade die Trans-Oxymethylierungs-Reaktion mit gutem Grund auch als einen völlig diskreten Vorgang, also ohne Auftreten freien Formaldehyds, formulieren. Wie man die Transaminierung über die Zwischenstufe

einer Schiffschen Base deutet, brauchen wir bei der Trans-Oxymethylierung als erste Phase nur eine Kondensation zwischen dem *β-Oxoalkohol-System* (XIV) und dem *Akzeptormolekül*(XV) unter Wasserabspaltung zur *Methylen-Verbindung*(XVI) und als zweite Phase deren Hydrolyse an der zur Carbonylgruppe *α,β*-ständigen Kohlenstoff-Kohlenstoff-Bindung unter Bildung des *Ketons*(XVII) und des *Alkohols*(XVIII) anzunehmen. An einen solchen völlig diskreten Mechanismus wird man im Zellgeschehen zu denken haben, doch

XIV XV XVI XVII XVIII



sollen die möglichen Korrelationen zwischen diesem und der Krypto-Formaldehyd-Reaktion in einer späteren Veröffentlichung behandelt werden. Hier diskutieren wir ausschliesslich Vorgänge, die sich bei der Aufarbeitung biologischen Materials ausserhalb der Zelle abspielen können und dadurch unsere Schlüsse bezüglich der Genuität der isolierten Individuen unsicher machen. — Um eine Krypto-Formaldehyd-Reaktion handelt es sich bereits, wenn das *3,5,5-Trimethylol-dihydro-desoxy-patulinsäure-lacton*(I) in schwach basischem Milieu Formaldehyd abspaltet und dieser sich einfach disproportioniert oder mit dem nach der Abspaltung verbleibenden "Donatorrumpf" eine gekreuzte Cannizzaro-Reaktion eingeht. Es wäre natürlich durchaus denkbar, den Versuch so zu leiten, dass an Stelle der Disproportionierung eine Butlerow-Kondensation zu Zuckern eintritt. In diesem Falle übernimmt der Formaldehyd selbst die Funktion des Akzeptors. Wir dürfen nicht übersehen, dass eine Krypto-Formaldehyd-Reaktion in gleicher Weise das Donator- und das Akzeptormolekül mehr oder weniger tiefgreifend verändert. In unseren Versuchen mit Barytlösung fanden wir das als ursprünglicher Donator fungierende *Lacton*(I) — je nach der Dauer der Einwirkung — in abgebauter und darüber hinaus reduzierter Form wieder. — Beispiele für eine Krypto-Formaldehyd-Reaktion bzw. Trans-Oxymethylierungs-Reaktion mit *anorganischem Akzeptor* stellen die Umsetzungen des *Lactons*(I), der *Anhydro-ennea-heptose*(III) und des *Tetrols*(IV) mit neutraler *Dinatriumsulfit-Lösung* dar, bei denen der Formaldehyd primär als *Bisulfitverbindung* gebunden und eine äquivalente Menge Alkali freigemacht wird, die ihrerseits sekundär wieder einen anderen Reaktionsmechanismus des Formaldehyds auslösen könnte. Hierher gehören auch die Reaktionen mit *Alkalilaugen* und *Tollensschem Reagenz*, bei denen der abgespaltene Formaldehyd reduktive Effekte entfaltet. Beispiele für Krypto-Formaldehyd-Reaktionen zwischen organischen Molekülen stellen die Übertragungen von Formaldehyd aus dem *Lacton*(I) und der *Anhydro-ennea-heptose*(III) in schwach alkalischer und aus dem *β-Acetyl-äthylalkohol* in saurer und neutraler Lösung auf das *Dimedon* $C_8H_{12}O_2$ unter Bildung von *Methylen-bis-dimethyl-dihydroresorcin* $C_{17}H_{24}O_4$ dar. Diese Versuche verdeutlichen ausserdem den Unterschied zwischen einer einfachen *Trans-Oxymethylierung* und einer *Krypto-Formaldehyd-Reaktion*, indem hier der abgespaltene Formaldehyd im Akzeptor-System nicht mehr als Methylolgruppe erkennbar ist,

sondern als *Methylenbrücke* zwischen zwei Akzeptormolekülen in Erscheinung tritt. Darüber hinaus zeigen die Versuche, mit welcher Leichtigkeit der Formaldehyd aus einem *β-Ketonalkohol-System* unter geeigneten Milieubedingungen auf andere Systeme übertragbar ist. Sobald während der Aufarbeitung aus einem Donator-System Formaldehyd frei wird, muss man daher damit rechnen, dass er von begleitenden Systemen aufgenommen wird, was auch deutlich aus den Versuchen Finckes [17] hervorgeht, in denen er zeigte, dass Formaldehyd — einem Pflanzenbrei zugesetzt — "zerstört" oder "gebunden" wird. Zur Veranschaulichung dieser Verhältnisse wollen wir auf der Grundlage vorliegender experimenteller Daten und der der lebenden Zelle zu Gebote stehenden Möglichkeiten aus dem weiten Spektrum anderer Kombinationen mehr oder weniger willkürlich nur eine herausgreifen. Wir wählen das Beispiel der *Acetessigsäure*, die uns durch den aus der Tierphysiologie bekannten Vorgang der *β-Oxydation* geläufig ist und deren hier zu diskutierende spezielle Reaktionen auch durch die Arbeiten anderer Forscher experimentell hinreichend unterbaut sind. Wir dürfen davon ausgehen, dass dem Organismus die Reduktion der *Acetessigsäure* zum *β-Acetyl-äthylalkohol* möglich ist, und nehmen an, dass in einem gegebenen biologischen Material *Acetessigsäure*, sagen wir in Form ihres *Äthylesters*, und *β-Acetyl-äthylalkohol* zusammen mit anderen Substanzen vorlägen. Durch das Experiment wissen wir, dass dieser als Methylolgruppen-Donator fungieren kann. Andererseits wissen wir, dass Formaldehyd unter verschiedenen Milieubedingungen [18,19] mit Acetessigester zu reagieren vermag. Nach Rabe und Rahm [18] treten Formaldehyd und Acetessigester bereits bei Zimmertemperatur zum *Methylen-bis-acetessigester* zusammen, der durch geringe Mengen Piperidin in den *Methyl-cyclohexanol-on-dicarbonsäure-ester*, bei Einwirkung von Chlorwasserstoff oder bei der Wasserdampfdestillation in den *Ester* der *Methyl-cyclohexen-on-dicarbonsäure* [19] übergeht. — An Stelle der als genuin angenommenen Verbindungen *β-Acetyl-äthylalkohol* und *Acetessigester* würden wir also bei Eintritt einer *Krypto-Formaldehyd-Reaktion* während der Aufarbeitung als Donatorrumpf das *Aceton* und als Akzeptorsystem ziemlich komplizierte *hydroaromatische Verbindungen* erhalten.

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Pyrazole Studies

IX *. The Kinetics of Oxidation by Oxygen of 1,3,4-Substituted Pyrazol-5-ones in the Presence of Hydroxyl or Alkoxyl Ions

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1,3,4-Substituted pyrazol-5-ones are, in alcoholic solutions containing equivalent or excess amount of sodium hydroxide, oxidised by oxygen, one half molecule of oxygen being consumed per mole of pyrazolone. The order of the reaction is dependent on the solvent and on the substituents present. When the concentration of oxygen is kept constant a zero order scheme is usually followed but in some cases a first order scheme, in others more complicated schemes are followed.

The yield of 4-hydroxysubstituted pyrazol-5-one is usually almost quantitative, independent of the order of the reaction.

In a previous paper (Veibel and Linholt [1]) was shown that 4-alkylsubstituted pyrazol-5-ones dissolved in methanol are oxidised to 4-alkyl-4-hydroxysubstituted pyrazolones by oxygen in the presence of triethylamine (which is at the same time oxidised to triethylamine oxide), one mole of oxygen being consumed per mole of pyrazolone.

We now found that this oxidation also takes place when an inorganic base such as the hydroxide ion or an organic base such as the ethanolate ion is present, but as these bases are not oxidised themselves the consumption of oxygen is only one half mole per mole of pyrazolone.

The oxidation of pyrazolones substituted at N_1 in the presence of triethylamine follows a first order scheme [1]. In the presence of the above named bases in alcoholic solution, more or less water being present, the oxidation usually follows a zero order scheme (care being taken that the concentration of oxygen is kept constant during the reaction), the velocity of consumption of oxygen being independent of the concentration of pyrazolone. The velocity is, however, strongly dependent on the alcohol used as solvent (investigated were methanol and *tert.* butanol) and the amount of water present.

* VIII: *Acta Chem. Scand.* **8** (1954) 1383.

Table 1, No. II, shows that for a particular pyrazolone the type of the reaction may shift from first order to zero order when *tert.* butanol is used instead of methanol or when the water content of the solvent (methanol) is reduced from some 10 % to some 2 %, but Table 1, No. VIII, shows that the inverse shift of reaction order is possible too.

The yield of the hydroxy compound isolated is practically independent of the order of reaction and the velocity of oxidation.

It seems inevitable to admit that the mechanism of the reaction in the presence of hydroxyl or ethanolate ions is different from the mechanism followed in the presence of tertiary amines. For the latter reaction we assumed the scheme:

$$PyRH + (C_2H_5)_3N \rightleftharpoons PyR^- + (C_2H_5)_3\overset{+}{N}H \qquad (a)$$

$$PyR^- + O_2 \longrightarrow PyROO^- \qquad (b)$$

$$2\ PyROO^- + 2\ (C_2H_5)_3\overset{+}{N}H \longrightarrow PyROH\text{---}O\text{---}HORPy + (C_2H_5)_3NO$$
$$\uparrow$$
$$N(C_2H_5)_3$$

$$PyROH\text{---}O\text{---}HORPy + H_2O \longrightarrow 2\ PyROH + (C_2H_5)_3N(OH_2)$$
$$\uparrow$$
$$N(C_2H_5)_3$$

PyRH meaning a 4-monoalkyl- or arylsubstituted pyrazol-5-one, at the same time alkyl- or arylsubstituted at C_3 and at N_1.

In the presence of sodium hydroxide the reaction presumably proceeds through the same initial stages (a) and (b) as above. But as water cannot be oxidised as could the triethylammonium ion another sequence of reactions starts. The possibility first considered was:

$$PyROO^- \rightarrow PyRO^- + 1/2\ O_2 \qquad (c_0)$$

but presumably a first order scheme would result for the overall reaction. A radical mechanism being indicated by the zero order scheme followed, we then considered the sequence:

$$PyROO^- \longrightarrow PyR\cdot + \cdot OO^- \qquad (c_1)$$
$$\cdot OO^- + PyRH \longrightarrow HOO\cdot + PyR^- \qquad (d_1)$$
$$HOO\cdot + PyRH \longrightarrow PyR\cdot + H_2O + 1/2\ O_2 \qquad (e_1)$$
$$PyR\cdot + O_2 \longrightarrow PyROO\cdot \qquad (f_1)$$
$$PyROO\cdot + PyR^- \longrightarrow PyRO\cdot + PyRO^- \qquad (g_1)$$
$$PyRO\cdot + PyR^- \longrightarrow PyR\cdot + PyRO^- \qquad (h_1)$$
$$PyRO^- + Na+ \longrightarrow PyRONa \qquad (i_1)$$

The chain is fully established in the reactions $(f_1) \rightarrow (g_1) \rightarrow (h_1)$. A chain breaking reaction would be $PyR\cdot + PyR\cdot \rightarrow RPy\text{—}PyR$ but we so far did not observe the formation of a bis-pyrazolone.

The initial reactions (a) → (b) → (c_1) → (d_1) → (e_1) might explain the existence of an induction period. In a few instances we observed a short period of induction but usually the zero order reaction was established at once.

In many instances we observed a consumption of oxygen somewhat greater than one half mole per mole of pyrazolone, possibly due to the formation of

O_2^{--} or to a further oxidation of the pyrazolone or the hydroxypyrazolone. We never succeeded in establishing the presence of hydrogen peroxide during the reaction.

In some instances the reaction follows a first order scheme whereas in other instances (mainly such pyrazolones where the oxidation proceeds slowly) a more complex reaction seems to take place, following neither a zero order, nor a first order nor a second order scheme. We have so far not succeeded in establishing the conditions determining the kinetics of the reaction. More accurate kinetic investigations are in preparation, including experiments with variation of the oxygen pressure.

The first order scheme is represented by the sequence:

$PyRH + R'O^-$	$\rightleftharpoons$	$PyR^- + R'OH$ ($R' = H$ or C_2H_5)	(a)
$PyR^- + O_2$	$\longrightarrow$	$PyROO^-$	(b)
$PyROO^- + PyR^-$	$\longrightarrow$	$2\ PyRO^-$	(c)
or $PyROO^-$	$\longrightarrow$	$PyRO^- + 1/2\ O_2$	(c')
$PyRO^- + Na+$	$\longrightarrow$	PyRONa	(d)

When (b) is slow as compared with the other reactions this reaction will determine the rate, *i. e.* at constant pressure of oxygen the consumption of oxygen will be proportional to the pressure of oxygen and the concentration of pyrazolone (or of pyrazolone ion):

$$\frac{dx}{dt_i} = k_1 \cdot p_{O_2} \cdot c_{py\text{-}}$$

On the other hand $c_{py\text{-}} = k_0 \cdot \frac{a-x}{a}$

where a is the volume of oxygen necessary for complete reaction and x the volume consumed at the time t. From this we get:

$$\frac{dx}{dt} = = k_1 \cdot p_{O_2} \cdot k_0 \cdot \frac{a-x}{a} = K_0 \cdot \frac{a-x}{a}$$

i. e. a first order scheme.

When (c') is followed instead of (c) the same expression holds good with a new value of the constant, but the (c) type of reaction is possibly more likely than the (c') type when regard is payed to the reaction between pyrazolones and *tert.* butylhydroperoxide described previously [2].

EXPERIMENTAL PART *

All pyrazolones investigated were prepared by current methods (for references see [1] and [3]). The 4-hydroxypyrazolones, too, have been recorded (Veibel and Linholt [2,3]) with the exception of the oxidation products of X and XI.

1,3-Dimethyl-4-hydroxy-4-phenylpyrazol-5-one (X ox). 1.9 g of 1,3-dimethyl-4-phenylpyrazol-5-one were dissolved in 20 ml of methanol, 2 ml of 5 *N* aqueous sodium hydroxide were added and the solution made up with methanol to 25 ml in a 100 ml flask. The air was expelled by oxygen and then the technique described previously [1] was applied.

* All m. p.'s uncorrected. Microanalyses by Mr. P. Hansen, Department of Organic Chemistry, University of Copenhagen.

Table 1. Oxidation of 1,3,4-trisubstituted pyrazol-5-ones by oxygen in different solvents.

Composition of solvent	2 ml 5 *N* NaOH in methanol to 25 ml	25 ml *N* aqueous NaOH		6 ml 2 *N* $NaOC_2H_5$ in *tert.* butanol to 25 ml		2 ml 5 *N* NaOH in *tert.* butanol to 25 ml		25 ml 2 *N* NaOH in methanol		25 ml 2 *N* aqueous NaOH		Yield %
mole base per mole pyrazolone	1.0	2.5		1.2		1.0		5.0		5.0		
	Order of reaction	Order of reaction	50 % oxidation	Order of reaction	50 % oxidation	Order of reaction	50 % oxidation	Order of reaction	50 % oxidation	Order of reaction	50 % oxidation	
I C_6H_5 CH_3 CH_3	1 18 h	—	—	—	—	—	—	—	—	—	—	90
II C_6H_5 CH_3 C_2H_5	1 9 h	—	—	0	35 h	0	4 h	0	11 h	—	—	90—100
III C_6H_5 CH_3 *n*-C_4H_9	— —	1	18 h	—	—	—	—	—	—	0	10 h	90
IV C_6H_5 CH_3 *iso*-C_4H_9	0 1.2 h	—	—	—	—	—	—	—	—	—	—	~100
V C_6H_5 CH_3 C_6H_{11}	0 0.3 h	—	—	—	—	—	—	—	—	—	—	~100
VI C_6H_5 CH_3 $C_6H_5CH_2$	complex ~200 h 50 %	—	—	—	—	—	—	—	—	—	—	~100
VII C_6H_5 *Cyclo*hexano	complex ~100 h 100 %	—	—	—	—	—	—	—	—	—	—	65
VIII CH_3 CH_3 C_2H_5	0 ~8 h	—	—	1	40 h	—	—	—	—	—	—	50
IX CH_3 CH_3 $C_6H_5CH_2$	0 60 h	—	—	0	70 h	—	—	—	—	—	—	~100
X CH_3 CH_3 C_6H_5	complex ~50 h 100 %	—	—	—	—	—	—	—	—	—	—	~100
XI C_6H_5 CH_3 C_6H_5	complex ~100 h 100 %	—	—	—	—	—	—	—	—	—	—	~40

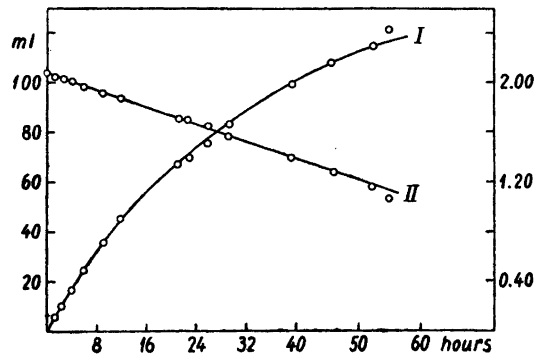

Fig. 1. 1-Phenyl-3,4-dimethylpyrazol-5-one.
I. Absorption of oxygen. (Ordinate left)
II. log (c−x)/x (Ordinate right)

The solution remained clear and colourless all during the oxidation. When no more oxygen was absorbed the flask was disconnected from the gas-burette, the sodium hydroxide was neutralised with hydrochloric acid and the solvents removed *in vacuo* (bath temperature ~50°), leaving the hydroxypyrazolone in nearly quantitative yield, m. p. 126°; recrystallised from aqueous ethanol (in order to remove sodium chloride) it was obtained as long needles with m. p. 126—127°. (Found: C 64.51; H 5.83; N 13.30. Calc. for $C_{11}H_{12}O_2N_2$ (204.2): C 64.68; H 5.93; N 13.72).

1,4-Diphenyl-4-hydroxy-3-methylpyrazol-5-one (XI ox). 12.5 g of 1,4-diphenyl-3-methylpyrazol-5-one were dissolved in 100 ml of methanol, 10 ml of 5 *N* aqueous sodium

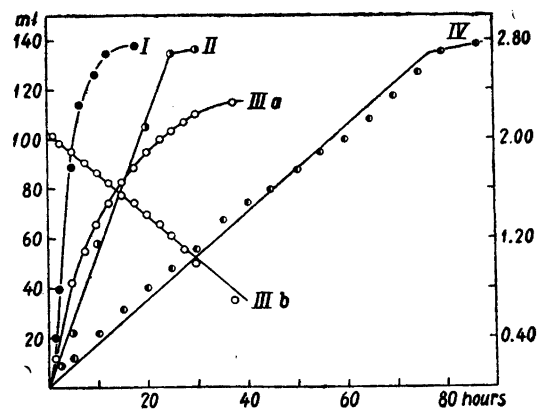

Fig. 2. 1-Phenyl-3-methyl-4-ethylpyrazol-5-one.
I. 0.01 mole + 2 ml 5 N NaOH diluted to 25 ml with tert. butanol. ●——●
II. 0.01 mole + 25 ml 2 N NaOH in methanol. ◑——◑
III a. 0.01 mole + 2 ml 5 N NaOH diluted with methanol to 25 ml. Absorption of oxygen (Ordinate left) ○——○
III b. As III a. log (c−x)/x (Ordinate right)
IV. 0.01 mole + 6 ml 2 N $NaOC_2H_5$ diluted to 25 ml with tert. butanol. ◐——◐

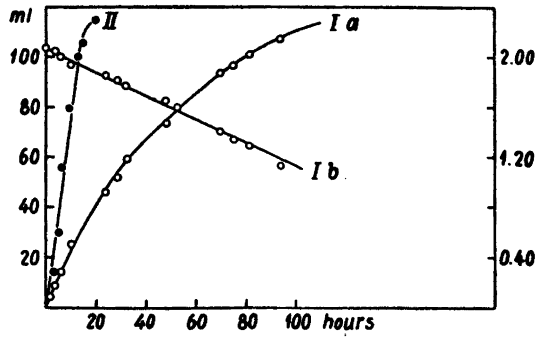

Fig. 3. 1,3-Dimethyl-4-ethylpyrazol-5-one.
I a. 0.01 mole + 6 ml 2 N $NaOC_2H_5$ + 14 ml tert. butanol, Absorption of oxygen (Ordinate left)
I b. As I a. log (c−x)/x (Ordinate right) O——O
II. 0.01 mole + 2 ml 5 N NaOH diluted to 25 ml with methanol. ●——●

hydroxide were added and the solution made up with methanol to 125 ml in a 500 ml flask. The procedure indicated above was followed for the oxidation. When no more oxygen was absorbed an excess of hydrochloric acid was added to the solution which had turned brown during the oxidation, and the solvents were removed *in vacuo* (bath temperature ~50°). A few ml of water were added to the solid residue to remove sodium chloride. The residue was isolated by suction and washed with water. Yield 6.5 g = 50 % of a coloured substance which was decolourised by treating its methanolic solution with absorption carbon; m. p. 135° which on repeated recrystallisations from aqueous methanol was raised to 141—143°. (Found: C 72.35; H 5.36; N 10.11. Calc. for $C_{16}H_{14}O_2N_2$ (266.3): C 72.15; H 5.30; N 10.52).

When 1,4-diphenyl-3-methylpyrazol-5-one was oxidised with *tert.* butylhydroperoxide as indicated by Veibel and Linholt [3] a hydroxy compound was obtained in good yield, showing m. p. 155—56° (Found: C 72.35; H 5.36; N 10.40. Calc. as above). A mixed m. p. of the two substances showed slight depression. Obviously the oxidation by oxygen is more complex than the oxidation with *tert.* butylhydroperoxide. Only about 60 %

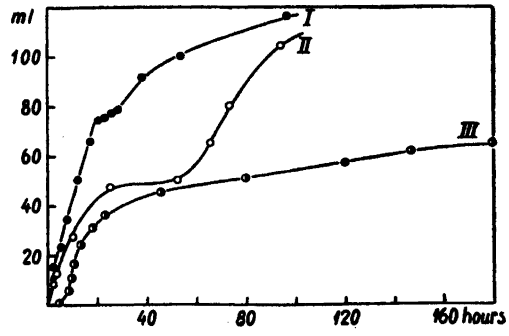

Fig. 4. I. 1-Phenyl-3-methyl-4-benzylpyrazol-5-one. ●——●
II. 1,3-Dimethyl-4-phenylpyrazol-5-one. O——O
III. 1,4-Diphenyl-3-methylpyrazol-5-one. ◑——◑

of the calculated volume of oxygen was consumed during 180 hours, no more oxygen being consumed during the next 80 hours. This pyrazolone is the only one investigated so far which does not consume at least one half mole of oxygen per mole of pyrazolone.

Oxidation of pyrazolones. Table 1 gives a summary of the results obtained.

F g. 1 (pyrazolone I) shows a typical example where a first order scheme is followed.

In Fig. 2 the results obtained with pyrazolone II are recorded. It is seen that only with a methanolic solution containing equivalent amounts of pyrazolone and sodium hydroxide and, in addition, *ca.* 10 % of water a first order scheme is followed (curve III), the oxygen consumed not exceeding the calculated amount. When *tert.* butanol is used as solvent instead of methanol (curves I and IV) or when the methanolic solution contains only 1—2 % of water, the ratio sodium hydroxide : pyrazolone being 5:1 (curve II), the reaction follows a zero order scheme and the consumption of oxygen exceeds the calculated volume by some 25 %. The velocity of the oxidation, too, is strongly dependent on the solvent used and the ratio base : pyrazolone. A more thorough investigation of the influence of these different factors is in preparation.

Fig. 3 shows that when pyrazolone VIII is oxidised a first order scheme is followed when the pyrazolone is dissolved in anhydrous *tert.* butanol, 1.2 moles of sodium ethanolate being present per mole of pyrazolone, whereas a zero order scheme is followed in methanol with 1 mole of sodium hydroxide, 10 % of water being present, *i. e.* the inverse of that found for the pyrazolone II.

Finally are in Fig. 4 recorded the oxidations of some pyrazolones where no definite scheme is followed (VI, X and XI). A closer investigation of the oxidation of these more complex cases is in preparation and no attempt to interpret the curves found here shall be ventured.

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Pyrazole Studies

X *. The Kinetics of Oxidation by Oxygen of 3,4-Substituted Pyrazol-5-ones in the Presence of Alkanolate Ions

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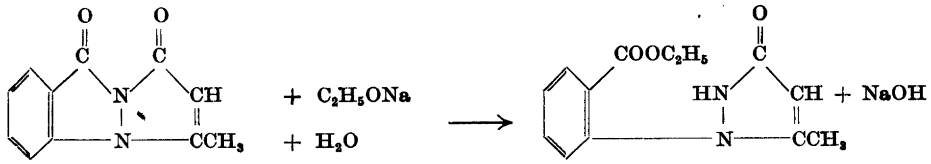
The oxidation by oxygen of pyrazol-5-ones unsubstituted at N_1 leads to unidentified oily oxidation products when water is present. In anhydrous solvents, however, the oxidation, catalysed by alkanolate ions, leads to 4-hydroxysubstituted pyrazol-5-ones, isolable in 80—92 % yield. The kinetics of the reaction is discussed.

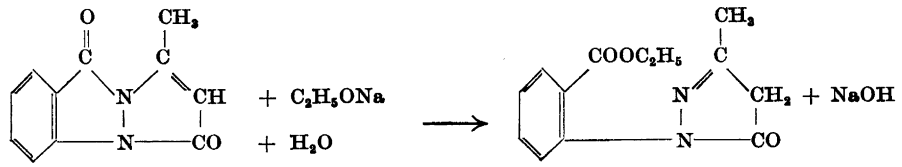
In previous papers was mentioned that the oxidation by oxygen of 3,4-substituted pyrazol-5-ones in the presence of triethylamine[2] or hydroxyl ions[1] cannot be adapted to the scheme followed by 1,3,4-substituted pyrazol-5-ones. In the presence of triethyl amine the absorption of oxygen starts as usual but it comes to an end before 1 mole of oxygen per mole of pyrazolone has been absorbed, and no definite reaction product could be isolated. In the presence of hydroxyl ions (or a mixture of hydroxyl and alkanolate ions) the amount of oxygen absorbed (1/2 mole of oxygen per mole of pyrazolone) is the same as for 1,3,4-substituted pyrazol-5-ones but no crystalline oxidation product can be isolated, only heavy oils which so far have not been identified. By analogy to some pyrazolones examined by Veibel and Lillelund [3] and by Veibel and Plejl [4] a rupture of the amidic linkage of the pyrazolone nucleus may be presumed:

Pyrazolones are usually described as substances resistant to both acid and alkaline hydrolysis. In the two substances mentioned the amidic linkage is weakened by the presence of the benzene nucleus condensed with the pyrazolone nucleus, in the present case the amidic linkage is weakened by the presence of the hydroxyl group. Further evidence is necessary, however, before this hypothesis, which has possibly significance for other 5-membered heterocycles too, can be regarded as proved.

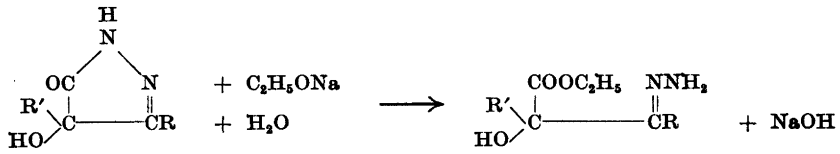
* IX: *Preceding paper.*



(Veibel and Lillelund [3])



(Veibel and Plejl [4])



In the absence of water, on the other hand, the 3,4-substituted 4-hydroxypyrazol-5-ones may be obtained in excellent yield by oxydation with oxygen of the 3,4-substituted pyrazol-5-ones in the presence of alkanolate ions, as they could by oxidation with *tert.* butylhydroperoxide [5]. When the 4-hydroxypyrazolones are treated with sodium hydroxide in ethanolic solution the crystalline substance is converted into oils similar to those isolated when the oxidation is carried out in solvents containing water.

EXPERIMENTAL PART

The pyrazolones investigated here and the resulting 4-hydroxypyrazolones have been described previously [5].

0.01 Mole of the pyrazolone was dissolved in 6 ml of 2 *N* sodium ethanolate and *tert.* butanol added to a total volume of 20 ml. The solution was shaken in an atmosphere of oxygen, the same technique as described previously [1] being applied. The volume of oxygen absorbed was noted at intervals.

After absorption of *ca.* 40 ml of oxygen (*ca.* 1/3 of the volume calculated for complete oxidation of the pyrazolone to a hydroxypyrazolone) the precipitation of a solid started without changing the rate of absorption of oxygen. When no more oxygen was absorbed 20 ml ether was added in order to complete the precipitation of the sodium salt which was isolated by suction, washed on the filter with ether or petroleum ether and dried by passing a stream of air through the filter cake, the salt not being hygroscopic. From the sodium salt the 3,4-substituted 4-hydroxypyrazolone was isolated as described previously [5].

Table 1 gives a summary of the pyrazolones examined and the results obtained. It is seen that whereas the N_1-substituted pyrazolones usually were oxidised following a zero order scheme [3], the N_1-unsubstituted pyrazolones are oxidised following a first order scheme or in a few cases a second order scheme. Figs. 1 and 2 show examples of these types.

Table 1. Oxidation of 3,4-substituted pyrazol-5-ones by oxygen in anhydrous solvents in the presence of ethanolate ions. 6 ml 2 N sodium ethanolate. Tert. butanol added to a total volume of 20 ml. 1.2 mole of base per mole of pyrazolone.

R	R^1	Order of reaction	Time for 50 % oxidation	Yield of sodium salt of the hydroxypyrazolone, %
CH_3	CH_3	1	8 h	15 *
CH_3	C_2H_5	1	6 h	90
CH_3	*n*-C_3H_7	2	4 h	92
CH_3	*iso*-C_3H_7	1	1 h	80
CH_3	C_3H_5	2	16 h	80
CH_3	*cyclo*-C_6H_{11}	1	1 h	90
CH_3	C_6H_5	2	27 h	85
CH_3	$C_6H_5CH_2$	1	17 h	80
C_6H_5	C_2H_5	1	3 h	80 *
C_6H_5	$C_6H_5CH_2$	1 **	27 h	80
	*Cyclo*hexano	1	6.5 h	80 *

* Yield of pure hydroxypyrazolone.
** Ethanol as solvent instead of *tert.* butanol.

DISCUSSION

Whereas the oxidation of N_1-substituted pyrazolones by oxygen in the presence of hydroxyl or ethanolate ions in many cases proceeded by a chain reaction [2] and the amount of oxygen absorbed often surpassed the calculated volume by 10—20 % [2], we here find no sign of a chain reaction and the absorption of oxygen approaches asymthotically the calculated volume.

In most cases we find $\log \frac{a-x}{a} = k \cdot t$ (a being the total amount of oxygen absorbed, x the volume absorbed at the time t), corresponding to a first order

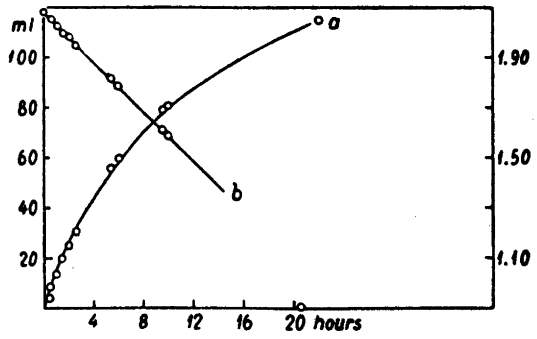


Fig. 1. 3-Methyl-4-ethylpyrazol-5-one. 0.01 mole + 6 ml 2 N $NaOC_2H_5$ diluted to 20 ml with tert. butanol.
a. Absorption of oxygen (Ordinate left)
b. log (c−x)/x (Ordinate right)

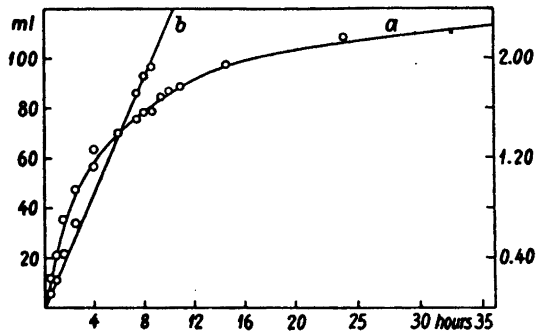


Fig. 2. 3-Methyl-4-propylpyrazol-5-one. 0.01 mole + 6 ml 2 N $NaOC_2H_5$ diluted to 20 ml with tert. butanol.
a. Absorption of oxygen (Ordinate left)
b. $x/(c-x)$ (Ordinate right)

reaction. In other cases we find $\frac{x}{a-x}=k\cdot t$, and this may be shown to correspond to a second order reaction.

When $\frac{x}{a-x}=k\cdot t$ we have:

$$x = a\cdot\frac{k\cdot t}{1+k\cdot t}$$

$$\frac{dx}{dt} = k\cdot a\cdot\frac{1}{(1+k\cdot t)^2} = k\cdot a\cdot\frac{1}{(1+\frac{x}{a-x})^2}$$

$$= k\cdot a\cdot(\frac{a-x}{a})^2$$

As $c_{Py^-} = k_1\cdot c_{Py_o^-}\cdot\frac{a-x}{a}$ we have $k_1\cdot\frac{a-x}{a}=\frac{c_{Py^-}}{c_{Py_o^-}}$

$$\text{or } \frac{dx}{dt} = k_2\cdot a\cdot\frac{(c_{Py^-})^2}{(c_{Py_o^-})^2} = K\cdot(c_{Py^-})^2$$

which is the expression for a second order reaction.

In the preceding paper we indicated a possible sequence of reactions leading to a chain reaction. Another possibility is:

$$PyRH + OR'^- \rightleftharpoons PyR^- + R'OH \quad (a)$$
$$PyR^- + O_2 \rightarrow PyROO^- \text{ or } PyR^- + O_2 \rightarrow (PyR^-, O_2) \quad (b)$$
$$2\ PyROO^- \rightarrow 2\ PyRO^- + O_2 \text{ or } 2\ (PyR^-, O_2) \rightarrow 2\ PyRO^- + O_2 \quad (c_1)$$
$$(PyR^-, O_2) + PyR^- \rightarrow 2\ PyRO^- \quad (c_2)$$
$$PyRO^- + Na^+ \rightarrow PyRONa \quad (d)$$

When (b) is slow as compared with (c) and (d) the result is a reaction following a first order scheme, provided the concentration (*i. e.* the partial pressure) of oxygen is kept constant. When on the other hand any of the reactions

(c_1) or (c_2) is slow as compared with (b) or (d) the result will be a reaction following a second order scheme. Some evidence may be produced in experiments with variation of the partial pressure of oxygen. By diminishing the partial pressure of oxygen it might be possible to change the order of reaction from 1 to 2. Experiments to this effect are planned.

It remains to be explained why a chain reaction will develop in pyrazolones substituted at N_1 but not in pyrazolones unsubstituted at N_1. The initial reaction steps are presumed identical for both types of pyrazolones. No simple explanation presents itself for the fact that in one case a chain reaction will develop, in the other not. An extension of the investigation to other 5-membered heterocycles may possibly throw light on this complicated problem.

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The Influence of the Solvent on Reaction Velocity

XII. The Solvolysis of Alkyl Benzenesulphonates in Acetone-Water and Dioxan-Water Mixtures

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Solvent effects in the uncatalysed hydrolysis of alkyl sulphonates show many similarities to those observed in the solvolysis of alkyl halides. The velocity of solvolysis of *iso*propyl benzenesulphonate is enormously increased by increasing the proportion of water in the medium, but that of ethyl, and of *n*-propyl, benzenesulphonates is affected to a much lower degree. In acetone-water and dioxan-water mixtures containing more than 50 % water, the reaction with the *iso*-propyl ester is of the sixth order but with the *n*-alkyl esters it is of the order 3.6—3.8 with respect to water. In media containing less water the order with respect to water is 2.5—1.7 with all three esters. The activation energies and frequency factors show great variations as the composition of the medium is varied. The reaction mechanism is discussed.

Parts X and XI of this series [1,2] dealt with the hydrolysis of alkyl halides in various acetone-water mixtures. The work described in the present paper [3] is an extension of the investigations on solvent effects in the field of nucleophilic substitution reactions. It also forms a continuation of four previous publications [4–7] on the hydrolysis of sulphonic esters, so that the experiments now cover the whole range of acetone-water and dioxan-water mixtures.

EXPERIMENTAL

Materials. The esters were obtained as described earlier [5]. However, it was found that when the alcoholate was used in about 10 % excess all the sulphonyl chloride reacted within an hour and that the heating on the water bath, and shaking with sodium hydroxide solution were unnecessary. Benzenesulphonyl chloride and the alcohols used for the syntheses were of the highest purity. The esters obtained were shown to be very pure by total hydrolysis. The acetone used as solvent in the kinetic experiments was Merck "acetone pro analysi".

All data concerning dioxan-water mixtures are taken from the paper of Tommila and Merikallio [6].

Rate measurements. Two different methods were used depending on the rate of the reaction. The experiments with *iso*propyl benzenesulphonate in water and in mixtures containing as little as 20 % acetone utilised the conductivity method described in previous work [1]. In all other cases the rate was followed by the titration methods previously described [4,5]. In media of lower water content than 20 %, where reaction is slow and rather high temperatures must be used, the kinetic experiments were carried out on 10 ml samples sealed in ampoules of Pyrex glass. In all these experiments the initial concentration of the ester was 0.05 *M*. In highly aqueous media more dilute solutions were used because of the low solubility of the esters, and the reactions were carried out in glass-stoppered vessels [4]. Since the reaction rate is somewhat dependent on the initial concentration of the sulphonic ester, all experiments in the same solvent were conducted using equal initial concentrations. In experiments of long duration at high temperatures the acetone became yellowish, but this did not seem to affect the reaction rate.

For the solvolysis, first-order kinetics were observed in all cases. From the variation of log k with $1/T$ the activation energy, E, and the frequency factor, A, were calculated by the method of least squares.

RESULTS AND DISCUSSION

The mechanism of the reaction. In the presence of a strong base the rates of hydrolysis of *n*-alkylbenzenesulphonates in 56 wt. % aqueous acetone can be expressed by the equation [7] $v = k_2[\text{ester}]\,[OH^-]$. The reaction is a bimolecular one-stage reaction or, according to the terminology of Hughes and Ingold, an S_N2 reaction:

$$RSO_2 \cdot O \cdot CH_2R' + OH^- \rightarrow [\overset{\sim\frac{1}{2}-}{RSO_2 \cdot O} \cdots \underset{R'}{CH_2} \cdots \overset{\sim\frac{1}{2}-}{OH}] \rightarrow RSO_2O^- + R'CH_2OH$$

(R=C_6H_5). Also the solvolysis presumably takes place by the same mechanism:

$$RSO_2 \cdot O \cdot CH_2R' + HOH \rightarrow [RSO_2 \overset{\delta-}{O} \cdots \underset{R'}{CH_2} \cdots \underset{H}{O} \cdots \overset{\delta+}{H}] \rightarrow RSO_2 \cdot O^- + RCH_2OH + H^+$$

The hydrolysis of *iso*propyl benzenesulphonate in aqueous acetone or in aqueous dioxan is independent of the presence of hydroxyl ions: its velocity is the same in alkaline, neutral or acid solution [5]. The rate-determining stage is the fission of the carbon-oxygen bond, the reagent intervening in a subsequent, rapid reaction ("unimolecular" or S_N1 mechanism):

$$RSO_2 \cdot O \cdot CH(CH_3)_2 \rightarrow [RSO_2 \overset{\delta-}{O} \cdots \overset{\delta+}{CH}(CH_3)_2] \rightarrow RSO_2O^- + \overset{+}{C}H(CH_3)_2 \quad \text{slow}$$

$$(CH_3)_2HC^+ + HOH \rightarrow (CH_3)_2HCOH + H^+ \quad \text{fast}$$

Sec.-butyl benzenesulphonate behaves similarly [5].

The *p*-toluenesulphonic esters are similar to the alkyl benzenesulphonates in their reactions [8]. Thus, the unimolecular mechanism appears to be more favoured for the reactions of sulphonic esters than for the analogous reactions of alkyl halides: secondary sulphonates generally resemble tertiary halides in behaviour.

The most economical mode of the reaction

$$X + YZ \rightarrow XY + Z$$

Table 1. The hydrolysis of ethyl benzenesulphonate in various acetone-water mixtures [1].

Acetone wt. %	$x_{acet.}$	10^7 k_1 sec.$^{-1}$						E cal.	log A
		50.00°	65.00°	75.00°	80.00°	90.00°	95.00°		
0	0	1 740						22 030	11.13
3.0	0.0095	1 610						21 800	10.95
7.0	0.023	1 290						21 410	10.60
17.8	0.063	745						21 370	10.32
36.5	0.151	257						21 730	10.11
56.0	0.283	86.8		974				21 730	9.64
80.0	0.553	18.2		201				21 460	8.78
90.0	0.736	5.67	23.7		86.6			20 600	7.69
92.5	0.793	3.50	14.4		51.5			20 300	7.29
95.0	0.855	2.80		27.3		97.0		20 430	7.26
96.5	0.896	(1.04) [2]	4.57		17.8	43.6 [3]		21 460	7.53
98.0	0.939	(0.286) [2]	1.42		6.20 [4]		24.9	23 000	8.00

[1] The values for 0—80 % acetone are taken from the paper of Tommila and Jutila.
[2] By the Arrhenius equation.
[3] 90.70°.
[4] 80.80°.

is usually that in which X is forced up against the repulsion of YZ and the bond between Y and Z is simultaneously stretched until X and Z can compete for Y on equal terms [9]. Thus we can regard the activation energy as being made up of two parts: that required to overcome the repulsion of the approaching reagent, and that required to weaken the existing bond [10]. One or the other of these might conceivably be the determining factor. The S_N2 mechanism is clearly analogous to the case in which the repulsion energy governs the situation. If the repulsion is large, it may require less energy to extend the Y—Z bond than to force X up to Y. The extreme case is that in which the bond between Y and Z is disrupted, after which X and Y combine; the mechanism is now S_N1. As we shall see, all three cases occur in the hydrolysis of *n*-alkyl sulphonates.

Table 2. The hydrolysis of n-*propyl benzenesulphonate in various acetone-water mixtures.*

Acetone wt. %	$x_{acet.}$	10^7 k sec.$^{-1}$					E cal.	log A
		40.00°	50.00°	60 00°	65.00°	75.00°		
0	0	334	986	2 650			21 500	10.52
13.0	0.044	220	627	1 570			20 370	9.57
27.2	0.104	94.8	265	657			20 070	8.99
50.0	0.237	20.8	60.8	167			21 580	9.39
56.0	0.283	16.3	48.2		217		21 800	9.42
80.0	0.553		9.02	23.8		99.4	21 500	8.49
90.0	0.736		2.68	7.12		27.0	20 650	7.40

Table 3a. The hydrolysis of isopropyl benzenesulphonate in highly aqueous acetone-water mixtures. Conductometric method.

Acetone wt. %	$x_{acet.}$	$[H_2O]$ mole/l	$10^5\ k_1$ sec.$^{-1}$						E cal.	log A
			25.00°	30.00°	35.00°	40.00°	45.00°	50.00°		
0	0	55.5		103	186	355	569	1 050	22 430	13.19
5.0	0.016	52.4			148	261	446	741	21 240	12.24
10.0	0.033	49.1		62.4	107	180		521	20 660	11.68
13.0	0.044	47.3		44.1	78.3	128	222	374	20 700	11.57
15.0	0.052	46.0	19.5	35.0	62.0	112	185	314	21 380	11.96
17.8 [1]	0.063	44.4	14.9			84.3		246	21 470	11.91
20.0	0.072	43.0	12.7	22.7	42.6	71.5		(204)[2]	21 600	11.91

[1] By the titration method.
[2] By the Arrhenius equation.

In the hydrolysis of sulphonic esters $RSO_2OCHR'R''$, the critical repulsion is that between the attacking reagent (OH^- or H_2O) and the central C atom of the alkyl group. If $R'' = H$, the repulsion is usually small and the mechanism S_N2; if R'' is an alkyl group, the repulsion is large and the reaction takes place by the S_N1 mechanism. Accordingly, S_N1 reactions should in general require higher activation energies than S_N2 reactions, for similar molecules. Also the frequency factor should be greater for an S_N1 than for an S_N2 process [1]. Both of these expectations are confirmed by our experiments (Tables 1—3, Figs. 1 and 2).

On passing from *n*-alkyl sulphonates to *iso*propyl benzenesulphonate the increase in E tends to reduce the reaction velocity but its influence is more

Table 3b. The hydrolysis of isopropyl benzenesulphonate in various acetone-water mixtures[1].

Acetone wt. %	$x_{acet.}$	$[H_2O]$ mole/l	$10^7 k_1$ sec.$^{-1}$						E cal.	log A
			40.00°	50.00°	65.00°	80.80°	90.00°	95.90°		
36.5	0.151	33.3	1 400	4 180					22 000	11.50
56.0	0.283	22.2	284	839					22 780	11.34
80.0	0.553	9.5	30.3	100					23 310	10.76
85.0	0.638	7.0	13.6	43.2	206				22 860	10.09
90.0	0.736	4.5	6.03	17.6	84.7				22 270	9.32
92.5	0.792	3.4		10.0	45.0	198			22 040	8.90
95.0	0.855	2.2		6.20	28.4		287 [2]		22 120	8.75
96.5	0.896	1.4		2.79	12.9	60.6			22 720	8.80
98.0	0.939	0.7		1.44	7.36		80.2	[3]	23 600	9.12
99.0	0.970	0.2		(0.906)[4]	4.93	25.9		106	24 600	9.60

[1] The data for 36.5—80.0 % acetone are taken from the paper of Tommila and Jutila.
[2] 90.50°.
[3] 98.38°: 176×10^{-7} sec.$^{-1}$.
[4] By the Arrhenius equation.

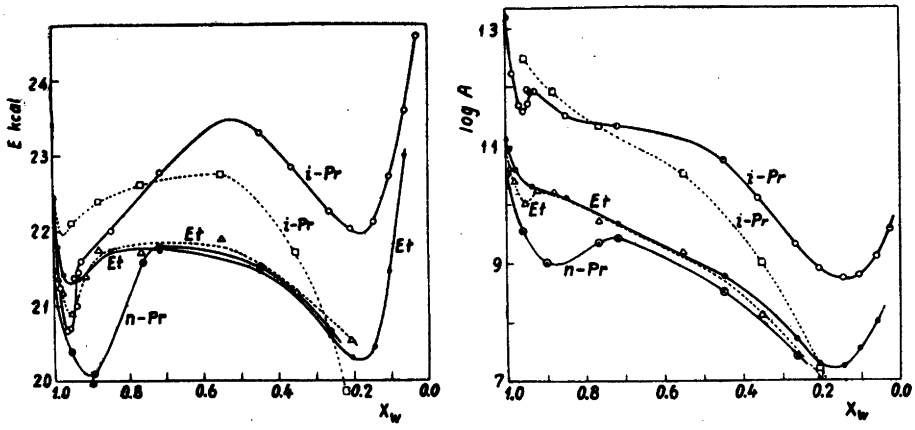
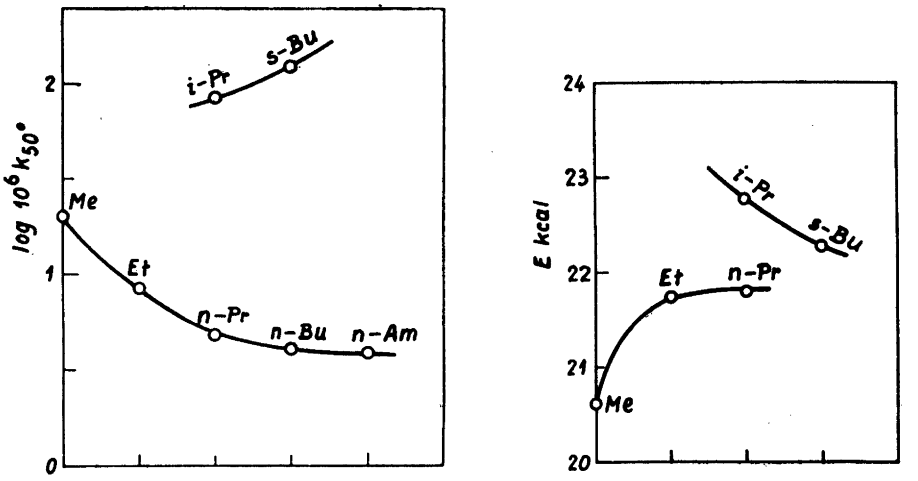


Fig.s 1 and 2. Variation of E *and log* A *with the composition of the solvent.* ——— *Acetone-water,* ····· *dioxan-water.*

than counterbalanced by the increase in A. The difference in mechanism manifests itself thus also in a much greater reaction velocity of the esters of the secondary alcohols. In addition, if the group —CHR'R" is varied in the direction of increasing negative charge on C, the velocity of a bimolecular reaction should decrease and that of a unimolecular reaction increase. In accordance with this, in 56 wt. % acetone where, as mentioned above, the *n*-alkyl esters undergo hydrolysis by the bimolecular and *sec.*-alkyl esters by the unimolecular mechanism the reaction rates follow the order Me > Et > *n*-Pr > *n*-Bu > *n*-Am$\langle\langle$$Me_2CH$ < EtMeCH (Fig. 3). The first-order rate-con-



Figs. 3 and 4. Velocities and activation energies of the hydrolysis (solvolysis) of alkyl benzenesulphonates in 56 wt. % aqueous acetone.

stant for *n*-amyl benzenesulphonate ($k_{50} = 40.0 \times 10^{-7}$ sec.$^{-1}$) was determined for this particular purpose, the other data are taken from previous investigations [7]. For *iso*butyl benzenesulphonate the reaction is very slow ($k_{50} = 4.79 \times 10^{-7}$ sec.$^{-1}$), partly owing to steric hindrance. The difference in mechanism is seen also from Fig. 4 which shows that the changes in the activation energy follow the course predicted by the theory.

The velocity of an S_N1 reaction should greatly increase with increasing ionising power of the solvent. In the bimolecular mechanism, too, passage from the initial state to the transition state involves development of charge and thus bimolecular reactions should also, although to a lesser degree, be favoured by increasing polarity of the solvent. These predictions have been verified in the solvolysis of alkyl halides and they are obeyed also in the solvolytic reactions of alkyl sulphonates. In pure water at 50° the velocity of the solvolysis of *iso*-propyl benzenesulphonate is 4.3 times as fast as in 17.8 wt.% acetone, 1 000 times as fast as in 80 wt.% acetone, 10 000 times as fast as in 92.5 wt.% acetone, and 73 000 times as fast as in 98 wt.% acetone. The corresponding numbers for ethyl benzenesulphonate are 2.3, 96, 500, and 6 080, respectively. The ratios for *iso*propyl benzenesulphonate are considerably smaller than the corresponding values for tertiary halides, whereas for ethyl benzenesulphonate they are much greater than for ethyl bromide [1].

In the previous work [1] it was found that for the solvolysis of alkyl halides the plot of log k against log $[H_2O]$ is linear in the region from pure water to about 60—70 % acetone. From the slope of the graph it was deduced that for *tert.*-butyl chloride about 7 water molecules, and for *tert.*-butyl bromide about 6 water molecules, are included in the formation of the transition state whereas in the case of ethyl bromide their number is only about 2. Using the same method, *viz.* writing

$$dx/dt = k_1(a-x) = k' \ [H_2O]^n \ (a-x)$$

whence

$$\log \ k_1 = n \ \log \ [H_2O] + \text{const.}$$

we obtain from Figs. 5 and 6 the following values for the critical number, n, of water molecules needed for the formation of the transition state:

*Iso*propyl benzenesulphonate:	0—45 % acetone	$n = 6.0$
	45—90 % »	$n = 2.5$
	90— »	$n = 1.7$
	0—50 % dioxan	$n = 6.0$
	50—90 % »	$n = 2.6$
Ethyl benzenesulphonate:	0—50 % acetone	$n = 3.8$
	50— »	$n = 1.7$
	0—50 % dioxan	$n = 3.6$
	50—95 % »	$n = 2.1$
n-Propyl benzenesulphonate:	0—50 % acetone	$n = 3.8$
	50— »	$n = 1.8$

As in the case of alkyl halides the mixtures clearly fall into two distinct groups with respect to the value of n. In the region from pure water to 45 wt. % acetone or 50 wt. % dioxan $n = 6.0$ for *iso*propyl benzenesulphonate.

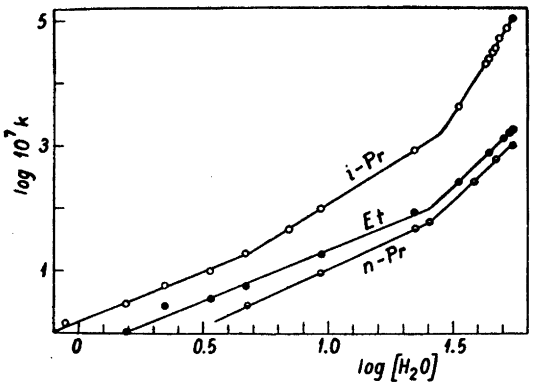


Fig. 5. Plot of log k *against log* $[H_2O]$. *Acetone-water, 50°.*

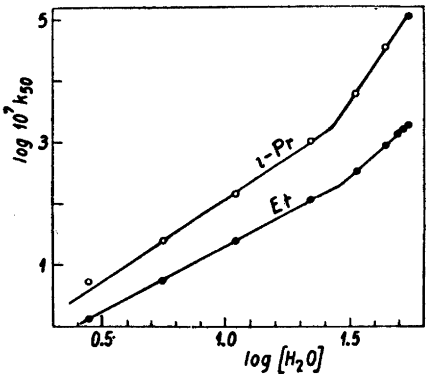
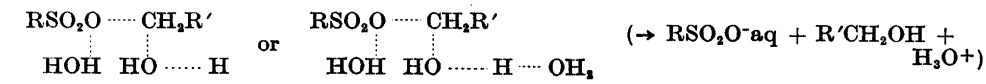


Fig. 6. Plot of log k *against log* $[H_2O]$. *Dioxan-water, 50°.*

As n is about 6 for t-BuBr and about 7 for t-BuCl the values $n = 6$—7 seem to be characteristic of S_N1 reactions. The values 2.1 to 1.7 found for n-alkyl benzenesulphonates in media containing less water than 50 wt. % agree with those found for ethyl bromide in acetone-water mixtures: obviously this order of magnitude is characteristic of S_N2 reactions.

The value $n \approx 2$ indicates that in the one-stage reaction one additional water molecule, besides the attacking molecule, is involved in the formation of the transition state. In the rate-determining step one water molecule forms an incipient bond to carbon and another becomes bound by a hydrogen bond to the O atom of the group O—C. Since in some media, and especially for the solvolysis of ethyl bromide [1], n is somewhat greater than two it is reasonable to assume that a third water molecule is involved in the transition state. This third molecule would be used to accept the proton from the attacking water molecule. Thus the transition state may be represented by (*cf.* Ref.[11])



In the case of S_N1 reactions, as the value of n shows, six water molecules must contribute the solvation energy necessary for the splitting of the carbon-oxygen bond. The carbonium ion is formed within an aqueous solvation shell, and ends its life by reacting with one of these water molecules (*cf.* Ref.[1]), a conclusion previously reached by Benfey, Hughes, and Ingold [8,12].

The values $n = 3.6$ to 3.8 found for the n-alkyl benzenesulphonates in the region between pure water and 50 % acetone or dioxan are distinctly greater than those deduced above for S_N2 reactions. Values of this order of magnitude do not exist in the solvolysis of ethyl bromide and they obviously suggest that in highly aqueous solvents n-alkyl benzenesulphonates have a tendency to change mechanism from an S_N2 to S_N1 reaction. Such a conclusion is supported

Table 4. The hydrolysis of ethyl benzenesulphonate in water at 40°; a = 0.01.

Time, min.	[NaOH] = 0		[NaOH] = 0.01			[NaOH] = 0.02		
	Change %	$10^5 k_1$	Change %	$10^3 k_2$	$10^5 k_1$	Change %	$10^3 k_2$	$10^5 k_1$
60	18.0	5.50	20.0	6.95	6.18	20	6.54	6.20
90	25.0	5.33	28.5	7.35	6.20			
120	33.0	5.56	36.8	8.32	6.36			
180	44.5	5.45	48.5	8.70	6.14	52.0	8.02	6.79
210	49.5	5.42	53.5	9.12	6.08	57.0	8.06	6.73
240	55.0	5.55	59.0	10.0	6.19	62.0	8.28	6.71
270	60.0	5.66	62.5	10.2	6.06	66.5	8.52	6.73

Table 5. The hydrolysis of ethyl benzenesulphonate in 7.0 wt. % acetone. Temperature 40.00°; a = 0.01, [NaOH] = 0.01.

Time, min.	70	100	120	150	180	210	240	360
Change %	18.5	25.0	28.9	34.7	37.3	42.6	47.2	61.1
$10^3 k_2$	5.40	5.52	5.62	5.88	5.52	5.87	6.20	7.27
$10^5 k_1$	4.87	4.79	4.73	4.73	4.32	4.40	4.44	4.37

(In absence of alkali $k_1 = 4.65 \times 10^{-5}$ sec.$^{-1}$)

Table 6. The hydrolysis of ethyl benzenesulphonate in 17.8 wt. % acetone. Temperature 25.00°; a = 0.02, [NaOH] = 0.02.

Time, hours	3	8	19 ½	24	27	33	43	50
Change %	5.5	13.7	30.5	36.2	40.0	46.5	54.5	59.4
$10^4 k_2$	2.70	2.76	3.16	3.27	3.43	3.66	3.88	4.07
$10^6 k_1$	5.22	5.12	5.23	5.20	5.25	5.26	5.09	5.00

(In absence of alkali $k_1 = 4.58 \times 10^{-6}$ sec.$^{-1}$).

Table 7. The hydrolysis of ethyl benzenesulphonate in 30 % acetone. Temperature 25.00°; a = 0.02, [NaOH] = 0.02.

Time, hours	21	24	32 1/4	43	48	70
Change %	22.0	24.8	31.7	40.0	43.4	55.0
$10^4 k_2$	1.85	1.91	1.98	2.15	2.21	2.42
$10^6 k_1$	3.29	3.30	3.28	3.30	3.29	3.17

Table 8. The hydrolysis of ethyl benzenesulphonate in 36.5 wt. % acetone. Temperature 25.00°; a = 0.05, [NaOH] = 0.05.

Time, hours	21	24	43	50	69	96
Change %	15.5	17.5	28.8	32.0	41.2	51.0
$10^4 k_2$	1.20	1.22	1.31	1.31	1.40	1.50
$10^6 k_1$	2.23	2.23	2.70	2.14	2.14	2.07

(In absence of alkali $k_1 = 1.51 \times 10^{-6}$ sec.$^{-1}$)

by the fact that in highly aqueous solvents first order kinetics were followed even in the presence of alkali (Tables 4—8). Reasons for the small increase of reaction velocity in the presence of alkali will be mentioned later. On the other hand, one is not justified in assuming that the mechanism is S_N1 since in that case n should be about six and the reaction velocity of the n-propyl ester should be greater than that of the ethyl ester. This is not the case. Thus we must conclude that the solvolysis of n-alkyl esters in highly aqueous solvents takes place in the following way. The introduction of a methyl group into ethyl benzenesulphonate increases the repulsion on the hydroxyl group in the approaching water molecule and causes the sulphonyl group to be held less tightly. The fact that the activation energy is lowered (Fig. 1) shows that the change in the bond energy governs the trend of the activation energy. In the transition state we have thus a relatively great bond streching, the entropy of activation being less negative (A greater) for ethyl benzenesulphonate than for n-propyl benzenesulphonate. The fact that the reaction is of the order 3.6—3.8 with respect to water indicates that four water molecules are intimately included in the formation of the transition state. The solvation of the alkyl benzenesulphonate molecule with these water molecules contributes the energy necessary for the stretching of the bond O—C between the sulphonyl and alkyl groups and after a sufficient extension of the bond one of the water molecules of the solvation layer can compete on equal terms with the sulphonyl group for the alkyl group. Thus the transition state is formed within an aqueous solvation shell of four water molecules and an exterior layer of less oriented water molecules. The observation that in highly aqueous solvents the hydrolysis of ethyl benzenesulphonate is almost unaffected by alkali, shows that the hydroxyl ion in general cannot penetrate through the solvation layer. Benfey, Hughes, and Ingold [12] reached the same conclusion when examining unimolecular substitution reactions, and they assume that the hydroxyl ion will take a proton from the outside of the solvation shell. The slight increase of the reaction velocity in our case is probably caused by a leakage of hydroxyl ions through the solvation layer or by normal salt effects, *cf.* Ref.[8]

In mixtures containing water less than 50 % by weight the hydrolysis of the two esters is powerfully accelerated by alkali and, as mentioned above, in 56 wt.% aqueous acetone the reaction is a second-order process [7]. For ethyl benzenesulphonate and n-propyl benzenesulphonate the activation energy is reduced in the presence of alkali and in alkaline and neutral solutions $E_{n-Pr} > E_{Et}$ (in the alkaline solution the values are 20 950 and 20 800 cal, respectively). Thus the reaction proceeds by a normal S_N2 mechanism. The change in the behaviour of the esters in these predominantly organic solvents is not solely due to the weaker solvation of the transition state by water ($n \approx 2$), but also to the decrease of the dielectric constant of the medium.

Since the rate of the reaction for the *iso*propyl ester, at least in solutions containing up to 56 wt.% acetone is not affected by the presence of alkali [5] the mechanism must be S_N1. The great reduction in n in passing to solvents containing less water than 50 % by weight suggests that in these solvents the S_N1 reaction is less favoured. A similar change at much lower water percentages can be observed in the solvolysis of *tert.*-butyl chloride and bromide [1]. It is unfortunate that because of the insolubility of sodium hydroxide in mixtures

containing more than 60 % acetone or dioxan the reaction in the presence of strong alkali cannot be investigated in this region.

That the unimolecular mechanism is less favoured as the proportion of water in the solvent is decreased is also seen from the fact that the ratios k_{i-Pr}/k_{Et} and k_{i-Pr}/k_{n-Pr} decrease rapidly with decreasing water content, whereas the ratio k_{Et}/k_{n-Pr} remains nearly constant or increases slightly (Table 9). This is true also in the solvolysis of alkyl halides (Table 10), but in this case the absolute values of the ratios and their decrease are much greater. However, also at low water concentrations the reaction velocities of the *iso*propyl ester

Table 9. The ratios of the rate constants, 50° C.

Acetone, wt. %	0	13.0	17.8	36.5	56.0	80.0	90.0	95.0
k_{i-Pr}/k_{Et}	60		33	16	9.7	5.5	3.1	2.2
k_{i-Pr}/k_{n-Pr}	106	61			17	11	6.6	
k_{Et}/k_{n-Pr}	1.76				1.80	2.02	2.12	

Table 10. The solvolysis of alkyl halides, 40° C.

Acetone, wt. %	0	17.8	27.2	36.5	56	76.7	85
k_{t-BuCl}/k_{EtBr}	88 500	18 100	8 500	4 600	870	190	
k_{t-BuBr}/k_{EtBr}					34 600	8 000	6 000

and of the tertiary halides are considerably greater than those of the *n*-alkyl compounds, whereas they should be smaller if the mechanism were S_N2.

Reaction velocity and the dielectric constant of the medium. The plots of log *k* against $(D—1)/(2D+1)$ (Fig. 7) or against *D* (Fig. 8) are of about the same form as those for the hydrolysis of alkyl halides. It is remarkable that in acetone-water the plot of log *k* against *D* is linear in the region from pure water to about 80 wt.% acetone. The slopes of the graphs for *iso*propyl benzenesulphonate are clearly greater than those for the *n*-alkyl esters. Similarly, in the solvolysis of alkyl halides the slopes of the curves for *tert.*-butyl bromide and

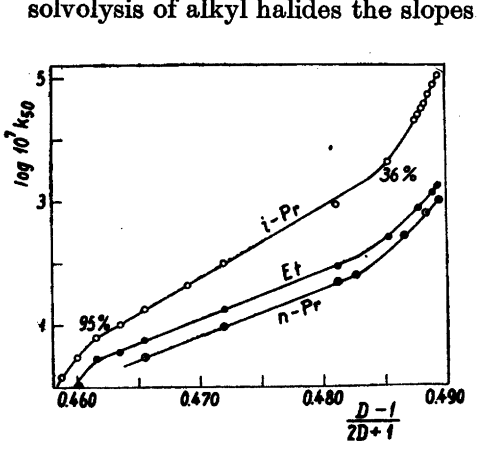


Fig. 7. Plot of log k *against* (D−1)/(2D+1). *Acetone-water, 50°.*

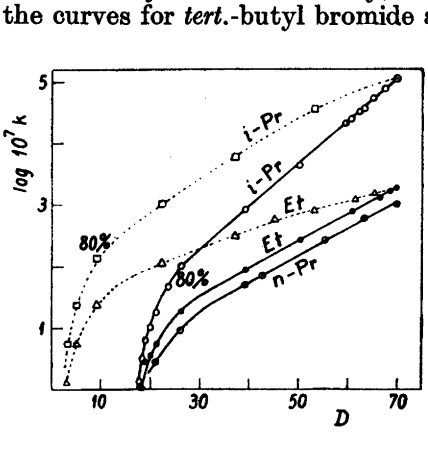


Fig. 8. Plot of log k *against* D, *50°.* —— *Acetone-water,* ····· *dioxan-water.*

chloride are greater than that for ethyl bromide. In solvents containing only a few per cent of water the slopes of the curves approach each other.

Parameters of the Arrhenius equation. The plots of E and log A against the composition of the solvent (Figs. 1 and 2) have about the same general shape as in the case of alkyl halides [1]. For all three esters, the second minimum appears for the same composition of acetone-water mixtures, $x_w \approx 0.18$, whereas for alkyl halides great variations occur in its position. Greater differences between the curves in acetone-water and dioxan-water appear for the solvolysis of *iso*propyl benzenesulphonate, whereas for ethyl benzenesulphonate the curves lie close together. The great difference in E and A between *iso*-propyl benzenesulphonate and *n*-propyl benzenesulphonate throughout the whole set of acetone-water mixtures, and between ethyl benzenesulphonate and *n*-propyl benzenesulphonate in highly aqueous solvents, is especially noticeable.

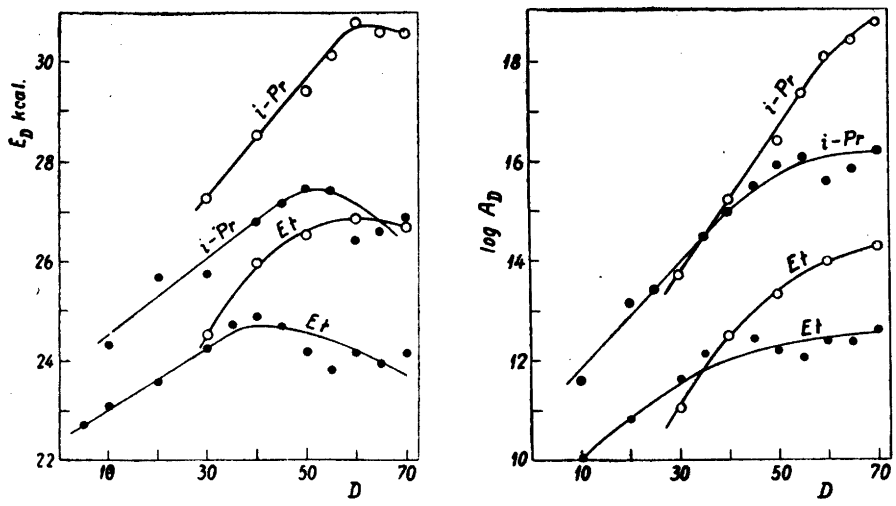
The dependence of E and A on the composition of the mixture used as solvent can be discussed in terms of the solvation of the initial state and of the transition state and of changes in the internal structure of the solvent. This has been described in a previous paper [1]. Especially noteworthy is the deep minimum in highly aqueous solvents, which indicates that the transition state is highly solvated and much more polar than the initial state.

Isodielectric solutions. The rate constants for isodielectric solutions, k_D, were obtained from the k's of Tables 1—3 and those given in Ref.[6] by graphical interpolation [1]. Plots of log k_D against $1/T$ are linear within the experimental error. The isodielectric activation energies, E_D, and frequency factors, A_D, are given in Table 11.

The dependence of E_D and A_D on D is illustrated in Figs. 9 and 10. For comparison it may be mentioned that the plots of log A_D *vs.* D are linear for the

Table 11. Isodielectric activation energies and frequency factors.

D	Acetone-water				Dioxan-water			
	Ethyl benzene-sulphonate		*Iso*propyl benzene-sulphonate		Ethyl benzene-sulphonate		*Iso*propyl benzene-sulphonate	
	E, cal.	log A	E, cal.	log A	E, cal.	log A	E, cal.	log A
70	26 660	14.28	30 560	18.71	24 150	12.60	26 900	16.23
65			30 600	18.40	23 940	12.35	26 600	15.87
60	26 820	13.98	30 680	18.09	24 150	12.39	26 440	15.61
55			30 150	17.36	23 800	12.03	27 420	16.11
50	26 500	13.33	29 240	16.38	24 180	12.19	27 480	15.95
45					24 690	12.42	27 180	15.52
40	25 940	12.50	28 520	15.22	24 870	12.41	26 790	15.00
35					24 701	12.14	26 420	14.51
30	24 500	11.02	27 270	13.69	24 200	11.63	25 720	13.78
20					23 530	10.80	25 700	13.22
10					23 100	10.04	24 340	11.63



Figs. 9 and 10. Variations of E_D *and log* A_D *with* D. O *Acetone-water,* ● *dioxan-water.*

alkyl halides, the positive slope being very small for EtBr. The plots of $E_D^{\neq}$ *vs.* D are also linear, with a positive slope for *t*-BuCl and *t*-BuBr, but with a small negative slope for EtBr.

For *iso*propyl benzenesulphonate as well as for ethyl benzenesulphonate the isodielectric activation energies and frequency factors are much higher than the corresponding values for the solvents of constant composition. For *t*-BuCl and *t*-BuBr E_D and A_D are always much higher than are E and A for the constant composition. However, for EtBr the isodielectric activation energy is a little smaller in highly aqueous solvents, but slightly greater in other solvents than that for constant composition. For EtBr A_D is only somewhat greater than A.

The relationship between log A_D and E_D is linear (Fig. 11) for $D<60$. The ratio Δ log $A_D/\Delta E_D$ is equal, *viz.* 1.30, for both esters and both sets of mixtures. A linear relationship between log A_D and E_D was found also for alkyl halides in acetone-water mixtures [1], the values of Δ log $A_D/\Delta E_D$ being: *t*-BuCl 2.4, *t*-BuBr 1.9, EtBr ≈ -1.

For ethyl bromide and the tertiary halides the difference in mechanism thus manifests itself very clearly in the isodielectric activation energies and activation entropies, whereas there are no so great differences in the behaviour of the isodielectric quantities between ethyl benzenesulphonate and *iso*propyl benzenesulphonate. The behaviour of E_D and A_D for the latter resembles closely that for the tertiary halides, and the behaviour of E_D and A_D for ethyl benzenesulphonate is little different.

Conclusions concerning the reaction mechanism. Summing up, it can be said that judging from solvent effects the mechanism of the hydrolysis of alkyl halides and sulphonic esters can be interpreted in terms of the general theory put forward by Hinshelwood, Laidler and Timm [10]. According to this theory

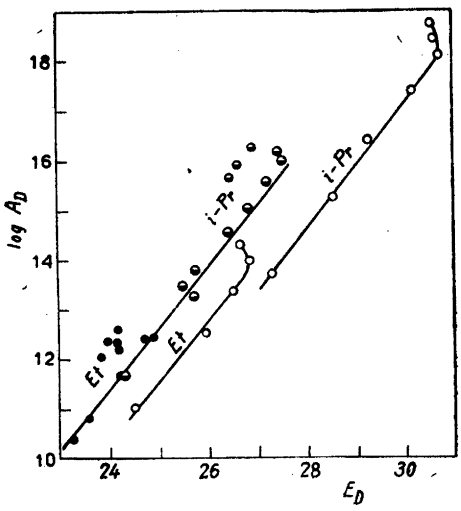


Fig. 11. Plot of log A_D *against* E_D. ○ *Acetone-water,* ◒, ● *dioxan-water.*

there is a graded series of mechanisms, with S_N1 and S_N2 as the extremes. All observations substantiate the view that a borderline reaction need not take place by two distinct (S_N1 and S_N2) and concurrent mechanisms, but only by one mechanism of intermediate type.

In all aqueous solvents ethyl bromide is hydrolysed by an S_N2 mechanism. The tertiary halides undergo hydrolysis by an S_N1 mechanism, but in media where the proportion of water is very small the conditions for the unimolecular mechanism are less favourable than in more aqueous solvents. Ethyl benzenesulphonate and *n*-propyl benzenesulphonate react in highly aqueous solvents by an intermediate mechanism; in solvents where the proportion of water is less than about 50 % by the bimolecular mechanism S_N2. *Iso*propyl benzenesulphonate undergoes hydrolysis by the unimolecular mechanism, but in solvents where the proportion of water is small this mechanism is greatly impeded.

These conclusions are essentially in agreement with the suggestions of Swain *et al.* [11,13], Winstein, Grunwald and Jones [14], von Doering and Streitwieser [15], and Hudson *et al.*[16], who have put forward arguments that there is a gradual transition from S_N2 to S_N1. This was also mentioned as a possibility by Hughes, Ingold *et al.*[17], but was not pursued. On their formulation, intermediate cases are interpreted as due to occurrence of simultaneous reactions involving the two mechanisms. Robertson in a recent investigation [18] has concluded that in the reaction $XC_6H_5SO_3R + Y \rightarrow XC_6H_5SO_3^- + RY$ (where Y is nucleophilic agent and X a *para* substituent) there are changes from S_N2 to S_N1, or *vice versa*, as the nucleophilic nature of Y, the character of R and the ionizing power of the solvent are changed.

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The Influence of the Solvent on Reaction Velocity

XIII. Alcoholysis of Sulphonic Esters

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The velocity of the alcoholysis of *iso*propyl benzenesulphonate is greater than that of ethyl benzenesulphonate in the same solvent. This is entirely due to the greater value of the frequency factor since the activation energy is greater for the *iso*propyl ester. For ethyl benzenesulphonate the mechanism of reaction is S_N2; for *iso*propyl benzenesulphonate an intermediate mechanism between S_N2 and S_N1. In pure dry methanol the mechanism for the latter approximates to S_N1. Addition of acetone or benzene to the alcohol used as solvent causes a decrease in E and A which is especially remarkable in the case of benzene. This decrease in E and A is produced by a breaking down of the internal structure of the alcohol.

The previous paper [1] of this series dealt with the hydrolysis of alkyl benzenesulphonates in various acetone-water and dioxan-water mixtures. For the purpose of further investigation into the solvent effect problem we have studied the solvolysis of alkyl benzenesulphonates

$$C_6H_5SO_3R + R'OH \rightarrow C_6H_5SO_3^- + ROR' + H^+$$

in certain binary non-aqueous mixtures. The reaction has been treated in two earlier papers [2,3] from this laboratory, but in pure alcohol only. The cases now investigated are ethyl benzenesulphonate in ethanol-acetone and ethanol-benzene mixtures and *iso*propyl benzenesulphonate in ethanol-benzene and in *iso*propanol-benzene mixtures.

EXPERIMENTAL

Chemicals. The esters were prepared as previously reported [1,3]. The alcohols used for the syntheses and as solvents in the kinetic experiments were commercial absolute ethanol and "alcool *iso*propylic pour analyse" of the firm U.C.B. Before use in the kinetic experiments they were dried: ethanol by the method of Walden [4], *iso*propanol by boiling with metallic calcium and distillation. The acetone was Merck's acetone "pro analysi"; it proved to be sufficiently dry for use as received. The benzene was a thiophene-free commercial product; before use it was dried with sodium and distilled.

Table 1. Alcoholysis of ethyl benzenesulphonate; ethanol-acetone mixtures.

EtOH ml/l	EtOH wt. %	x_{EtOH}	[EtOH] mole/l	10^6k sec.$^{-1}$ 40.00°	50.00°	60.00°	75.00°	E cal.	log A	ΔS^* E.U.
1 000*	100	1	17.1	1.52	4.27	12.9	49.2	21 670	9.303	−18.0
900	89.9	0.918	15.4	0.980	2.99	8.02	33.5	21 800	9.217	−18.4
800	79.5	0.830	13.7	0.703	2.17	6.02	22.5	21 450	8.883	−20.1
700	69.3	0.740	12.0	0.526	1.66	4.41	17.1	21 470	8.722	−20.6
600	59.3	0.647	10.3		1.13	3.10	12.6	21 600	8.664	−20.9
500	49.5	0.552	8.6		0.733	2.12	8.07	21 400	8.344	−22.4
400	39.6	0.453	6.9		0.474	1.35	5.03	21 060	7.929	−24.3
300	29.8	0.348	5.1		0.294	0.770	2.92	21 530	7.351	−26.9
200	19.9	0.238	3.4		0.141	0.399	1.55	21 380	7.616	−25.7

* Tommila and Lindholm [2]: E 22 000 cal., log A = 9.50.

Method. The kinetic experiments were carried out in sealed tubes of Pyrex glass. Into each tube 5 ml of a 0.05 M solution of the ester was measured by means of a pipette, the tubes (8—10 in each run) were then sealed and maintained in thermostatically controlled baths the temperature of which could be held constant within about ±0.02°. Tubes were removed after various periods of time, cooled quickly by immersing in a mixture of ice and water, and analysed by titration with 0.01 N baryta solution, cresol red being used as indicator. The rate constants were calculated by the first order formula $k = \frac{1}{t}\ln\frac{a}{a-x}$, which in all cases gave a good constancy for k.

The reaction between the *iso*propyl ester and sodium *iso*propylate was also investigated in pure *iso*propanol. Equal quantities (5 ml) of 0.05 M solutions of the reagents were mixed together in the reaction vessel [2] so that the initial concentration of each was 0.025 M. The reaction was stopped by adding a slight excess of 0.01 N HCl and the acid titrated with baryta solution. The velocity constants were calculated using the equation $k = x/ta(a-x)$.

Table 2. Alcoholysis of ethyl benzenesulphonate; ethanol-benzene mixtures.

EtOH ml/l	EtOH wt. %	x_{EtOH}	[EtOH] mole/l	10^6k sec.$^{-1}$ 40.00°	50.00°	60.00°	75.00°	E cal.	log A	ΔS^* E.U.
1 000	100	1	17.1	1.52	4.27	12.9	49.2	21 670	9.303	−18.0
900	88.8	0.944	15.4	1.12	3.29	9.47	38.0	21 870	9.314	−17.9
800	78.0	0.883	13.7	0.908	2.75	7.64	29.7	21 600	9.036	−19.2
700	67.4	0.817	12.0	0.723	2.17	6.04	23.2	21 500	8.866	−20.0
500	47.2	0.654	8.6		1.19	3.21	12.4	20 900	8.202	−23.0
300	27.6	0.449	5.1		0.536	1.40	5.01	19 960	7.228	−27.5
100	9.1	0.175	1.7			0.226	0.640*	17 150	4.590	−39.5
50	4.9	0.091	0.94			0.0667	0.177	15 100	2.728	−48.1

* 95.5°: 2.73×10^{-6}.

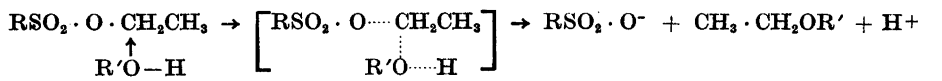
Table 3. Alcoholysis of iso*propyl benzenesulphonate;* iso*propanol-benzene mixtures.*

PrⁱOH ml/l	PrⁱOH wt. %	x_{PrOH}	[PrⁱOH] mole/l	10^6k sec.$^{-1}$ 40.00°	50.00°	60.00°	75.00°	E cal.	log A	ΔS^* E.U.
1 000	100	1	13.1	1.41	4.28	11.18	46.4	21 630	9.252	−18.2
900	89.0	0.911	11.8	1.02	3.08	8.46	33.1	21 520	9.033	−19.2
700	67.1	0.726	9.2	0.616	1.71	4.67	17.8	20 900	8.370	−22.2
500	46.9	0.534	6.6	0.292	0.827	2.22	8.35	20 750	7.945	−24.2
300	27.6	0.332	3.9		0.335	0.854	3.11	19 930	7.002	−28.5

The activation energy, E, and frequency factor, A, were calculated from the variation of log k with $1/T$, the method of least squares being used in all cases. The rate constants and the parameters of the Arrhenius equation are summarised in Tables 1—4.

RESULTS AND DISCUSSION

A comparison of Tables 2 and 4 shows that for *iso*propyl benzenesulphonate the reaction is about three times as fast as for ethyl benzenesulphonate in the same solvent. For *iso*propyl benzenesulphonate, however, the activation energy is clearly higher; thus its greater reaction velocity is entirely caused by a greater frequency factor. These results resemble those observed for the uncatalysed hydrolysis of these two compounds in acetone-water, and they suggest that for the ethyl ester the reaction mechanism is S_N2,



and for the *iso*propyl ester S_N1:

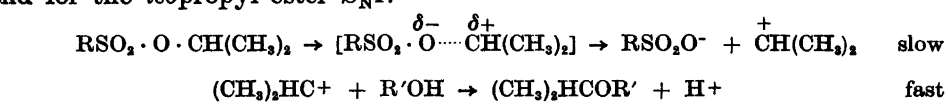


Table 4. Alcoholysis of iso*propyl benzenesulphonate; ethanol-benzene mixtures.*

EtOH ml/l	EtOH wt. %	x_{EtOH}	[EtOH] mole/l	10^6k sec.$^{-1}$ 40.00°	50.00°	60.00°	E cal.	log A	ΔS^* E.U.
1 000	100		17.1	4.18	12.8	38.6	23 000	10.690	−11.6
900	88.8	0.944	15.4	3.42	10.8	31.0	22 800	10.470	−12.6
700	67.4	0.817	12.0	2.14	6.55	18.5	22 350	9.927	−15.1
500	47.2	0.654	8.6	1.15	3.45	9.65	22 100	9.483	−17.1

As mentioned in the experimental section, we have also studied the reaction between the *iso*propyl ester and sodium *iso*propylate

$$C_6H_5SO_2OPr^i + Pr^iONa \rightarrow C_6H_5SO_2ONa + Pr^iOPr^i$$

in dry *iso*propanol. For 50° the results are (p is the percentage change, $a = 0.05$ moles litre):

t min.	120	205	240	300	360	390	
p	16.8	25.6	28.8	34.0	38.4	40.0	
10^4k_2 1 mole^{-1} sec.$^{-1}$	5.61	5.60	5.62	5.72	5.77	5.70	Mean: 5.67

As may be seen the second order law is obeyed. For the whole series of experiments the values obtained are:

	40.00°	50.00°	60.00°	E_{cal}	log A
10^4k_2 1 mole^{-1} sec.$^{-1}$	2.30	5.67	13.9	18 620	8.352

For the solvolysis of the *iso*propyl ester in pure *iso*propanol the following series was obtained at 50°:

t min.	845	1 080	1 500	1 680	1 800	1 980	2 040	
p	19.4	24.4	31.6	34.8	37.2	40.0	41.2	
10^6k_1 sec.$^{-1}$	4.25	4.32	4.22	4.24	4.31	4.30	4.34	Mean: 4.28

On a percentage basis the reaction with the alcoholate is about five times as fast as that with *iso*propanol suggesting an S_N2 mechanism. The concentration of *iso*propanol remains practically constant. The density of the alcohol is 0.788, and thus its concentration is 13.1 moles/litre. If the above value of k_1 is divided by the concentration we obtain for the bimolecular rate constant the value $k_2 = 3.27 \times 10^{-7}$ litres mole^{-1} sec.$^{-1}$.

In *iso*propanol and *iso*propanol-benzene mixtures the alcoholysis of *iso*propyl benzenesulphonate is slower than in ethanol and ethanol-benzene mixtures. However, the activation energy is greater in the ethanolic solutions but it is more than compensated by the frequency factor. It is probable that the lower values of E and A in the *iso*propanolic solutions are due to the greater effect of the solvation of the transition state.

If, in analogy with the treatment used in the case of the hydrolysis [1], it is assumed that the reaction rate is proportional to the nth power of the alcohol concentration,

$$\frac{dx}{dt} = k' \, [ROH]^n \, (a—x),$$

we can, since the concentration of the alcohol remains practically constant during the course of the reaction, put $k'[ROH]^n = k_1$, where k_1 is the measured first-order rate constant. Thus, $\log k_1 = n \log [ROH] + \text{const.}$, and a straight line of slope n should be obtained for the plot of $\log k_1$ against $\log [ROH]$. This is indeed the case (Fig. 1). For the *iso*propyl ester $n = 2.0$ in either series

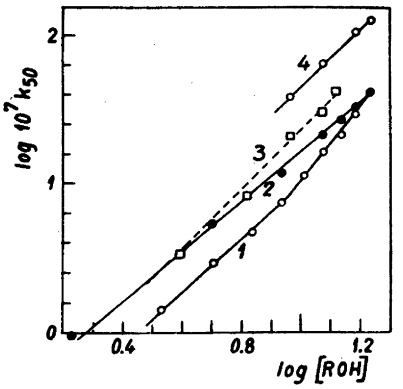


Fig. 1. Plot of log k *against log* $[H_2O]$. *Temperature 50°.*

1. Ethyl ester, ethanol-acetone
2. » » *ethanol-benzene*
3. Iso*propyl ester,* iso*propanol-benzene*
4. » » *ethanol-benzene*

of solvents. For the ethyl ester $n = 1.7$ in ethanol-benzene mixtures but in ethanol-acetone mixtures the graph has two linear parts with $n = 1.7$ and $n = 2.5$. In the solvolysis of alkyl benzene sulphonates in acetone-water or in dioxan-water, and in the solvolysis of alkyl halides in acetone-water, two or probably three water molecules are included in the formation of the transition state if the mechanism is S_N2, but if the mechanism is S_N1 their number is six or seven. However, in the alcoholysis of 1-halogenoethers where the reaction takes place by an S_N1 mechanism plotting of log k against log [ROH] has given straight lines with slopes from 3.2 to 5.2 in alcohol-dioxan mixtures, and from 1.5 to 2.0 in alcohol-benzene and alcohol-carbon tetrachloride mixtures [6].

It is obvious that for the alcoholysis of ethyl benzenesulphonate the mechanism is S_N2. For *iso*propyl benzenesulphonate the mechanism cannot be S_N2 since its alcoholysis is more rapid than that of ethyl benzenesulphonate in the same solvent. However, the following facts are not consistent with an S_N1 mechanism: (a) the reaction is accelerated by presence of alkali (see also Ref.[3]); (b) the increase of the reaction velocity with increasing proportion of ethanol in the solvent is about the same with the isopropyl ester as with the ethyl ester, whereas in acetone-water S_N1 reactions are much more influenced by changes in the composition of the solvent than are S_N2 reactions [1,5]; (c) the value of n is only about 2; (d) although the values of A are greater for *iso*propyl benzenesulphonate than for ethyl benzenesulphonate, they are still distinctly lower than those found for S_N1 reactions in acetone-water and in dioxan-water [1,5]. It is unlikely that the two mechanisms occur simultaneously. The kinetic relationships would then be complex. If the ester disappears in two simultaneous reactions, each with its individual energy of activation, the rate constants for various temperatures would hardly be expected to conform to a simple Arrhenius equation in all solvents.

As in the case of the water solvolysis of sulphonic esters a coherent interpretation of the experimental results can be given if, as suggested by Hinshelwood, Laidler, and Timm [7], we regard the activation energy as being made up of two parts: that required to overcome the repulsion of the approaching reagent (ROH) and that required to weaken the existing bond (O—CHR'R"), *i. e.*, to stretch it from its normal length to the length in the transition state.

In ethyl benzenesulphonate the carbon atom which is the reaction centre is only weakly negative; thus the repulsion is small and the mechanism is S_N2. In *iso*propyl benzenesulphonate the introduced methyl group enhances the repulsion of the hydroxyl group in the approaching alcohol while causing the sulphonyl group to be held less tightly. The fact that the activation energy is increased in ethanolic solutions shows that the change in repulsion is the most important. This view is supported also by the fact that in pure absolute methanol, the activation energy of the alcoholysis is greater for the *iso*propyl ester than for the ethyl ester, *viz.* 23 000 and 22 100 cal., respectively [2,3]. The most economical mode of reaction will then be that in which the alcohol molecule is forced up against the repulsion and the bond O—C in the ester is stretched at the same time until the alcohol molecule and the RSO_2O-group can compete on equal terms for the —$CHMe_2$ group. As the calculations of Eyring and Polanyi [8] show, such cases are very common. Thus the structure of the transition state will be less rigid, *i. e.* the entropy of activation less negative and the frequency factor greater than in the case of ethyl acetate in the same solvent. This is in accordance with the experimental results.

In methanol, where the kinetics in presence of alkali are between the orders one and two [3], the reaction very likely proceeds by a mechanism which approaches S_N1. This is obviously due to the fact that methanol is a more ionising solvent than are ethanol and *iso*propanol. Furthermore in methanol the ratio k_{Pr}/k_{Et} is greater than in ethanol, *e. g.* at 50° the values are 9.9 and 3.0, respectively.

A comparison of the parameters of the Arrhenius equation for the solvolysis in various pure solvents is given in Table 5. In each solvent E and A are higher for the *iso*propyl ester than for the ethyl ester, the difference increasing with the decrease of the ionizing power of the solvent and ΔE is 400, 900, and 1 330 calories in H_2O, MeOH, and EtOH, respectively. In water, where the solvolysis of the *iso*propyl ester proceeds by an S_N1 mechanism, its frequency factor is conspicuously high but high values of A are found also for the solvolysis of ethyl benzenesulphonate in water and for the alcoholysis of *iso*propyl benzenesulphonate in methanol, *i. e.* for cases where the mechanism is not "pure" S_N2.

With an increasing proportion of acetone or benzene in the solvent the activation energy and frequency factor decrease. This phenomenon is most

Table 5. The reaction $C_6H_5SO_2OR + R'OH \rightarrow C_6H_5SO_2O^- + H^+ + ROR'$ *in various pure solvents.*

R	Et		*i*-Pr	
Solvent	E cal.	log A	E cal.	log A
H_2O	22 030	11.3	22 430	13.19
MeOH	22 100	9.84	23 000	11.13
EtOH	21 670	9.30	23 000	10.69
i-PrOH			21 630	9.25

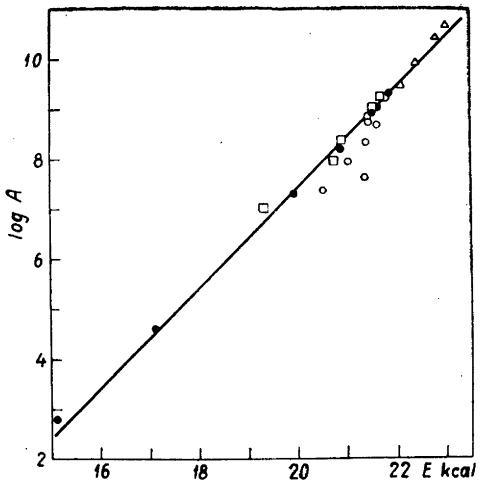


Fig. 2. Plot of log A *against* E. O *Ethyl ester, ethanol-acetone.* ● *Ethyl ester, ethanol-benzene.* △ Iso*propyl ester, ethanol-benzene.* □ Iso*propyl ester,* iso*propanol-benzene.*

simply explained by the change in the internal structure of the solvent. On addition of acetone or benzene to the alcohol the internal structure of the alcohol will be gradually broken down, the chains formed by the alcohol molecules being split to shorter chains and finally to single molecules distributed among the molecules of the added component. The alcoholysis of alkyl benzenesulphonates is a reaction of the type where the transition state is more polar and, consequently, more solvated than the initial state. Before the solvent can solvate the transition state the molecular bonds between the solvent molecules must presumably be broken and the heat of solvation is reduced by the amount of energy required to break these bonds. The decrease of E by solvation is therefore reduced but the further the splitting of the alcohol chains proceeds the greater will be the decrease. In ethanol-acetone mixtures there exist between the ethanol and acetone molecules weak hydrogen bonds which must in general be broken before the solvent can solvate the transition state; indeed, there is only a slight lowering of the activation energy with an increasing proportion of acetone. The formation of the mixture is endothermic in all three cases [9] but is much more so for the alcohol-benzene than for the alcohol-acetone mixtures. The variation of the activation energy is in good agreement with this state of affairs, which may explain much of the decrease of E with a decreasing proportion of alcohol in the solvent.

The more the internal structure of the pure alcohol is broken down the greater is the disorder of the molecules in the mixture and the greater will be the entropy decrease which the molecules of the solvent suffer in 'freezing' around the transition state; hence the decrease of A with the decrease of the proportion of the alcohol in the solvent. As in many other cases, there exists a linear relationship between log A and E (Fig. 2).

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Association Numbers in Liquid Systems from Intermolecular Free Length Relationships

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The association number x in a pure liquid, mixture or solution can be obtained from $x = (L_{poly}/L_{mono})^3$ if the size of the molecules in the system is of the same order of magnitude. L_{poly} is the average free length between the associated molecular aggregates and it can be obtained from experimental values for the compressibility (ultra sonic velocity). For pure liquids L_{poly} can also be obtained from the surface tension. L_{mono} is a hypothetical free length that would prevail in the system if no association occurred and the density were unchanged. It can be obtained from the thermal expansion of the liquid. From the value for the apparent critical temperature it is also possible to calculate relative association numbers with good accuracy in homologous series. The apparent critical temperature is a hypothetical critical temperature the liquid would have if the degree of association did not change with increasing temperature. It is easily obtained from thermal expansion data. For cluster associated systems, such as those composed mainly of alcohols, organic acids and other organic compounds, the association numbers obtained are accurate within a few tenths of an association number unit. The results calculated from the three methods agree within the limits of experimental error. For the aliphatic alcohols the association numbers decrease from 3.5 for methyl alcohol to 1.3 for octyl alcohol. Formic acid and acetic acid have the values 2.2 and 2.1, respectively. For lattice associated systems, such as water and dilute aqueous solutions, the order of magnitude of association might be obtained above a certain temperature.

It is customary to divide liquids into two classes, normal and associated, which have widely different properties [1–3]. In general associated liquids, such as water, alcohols and organic acids, have high melting and boiling points, large viscosities and high dielectric constants. They are generally miscible with one another, but the properties of the mixture are not simply related to those of the components. The explanation of these facts is that in such liquids there is a tendency for one molecule to be attached to a specific part of another. As a result of this the molecules tend to become associated into rings, chains or more complex aggregates.

Several accurate methods exist for the determination of molecular association in the gaseous phase [3,4]. For studies in liquid systems, and in particular pure liquids, many different methods have been suggested [4] but most of them yield results which are not in accord with each other, and have not been accepted for general use. In the present work some new methods will be described for studying association in both pure liquids and liquid mixtures. These methods have the advantage that the underlying principles are simple and that the results obtained from the various methods generally agree satisfactorily. The calculations are based on the employment of the concept of the free intermolecular length, which is related in a simple way to several other properties such as compressibility [5], surface tension [6], viscosity [7], and thermal expansion [8].

DEFINITIONS

The intermolecular free length in liquid systems can be defined in several ways using different molecular models [3,5,7,8]. When studying properties such as compressibility, surface tension and thermal expansion, which depend on molecular displacements in a direction perpendicular to the molecular surfaces, the molecules can be assumed to be spherical and having either a hexagonal or cubical packing. Under these assumptions, simple stereometric considerations give a molecular diameter

$$d = kV_0^{\frac{1}{3}} \tag{1}$$

where V_0 is the molar volume at absolute zero and $k = (2^{\frac{1}{2}}/N)^{\frac{1}{3}} = 1.329 \times 10^{-8}$ and N is Avogadro's number.

For a non-associated substance the diameter is unchanged when the temperature is raised, but the liquid expands as a whole as is illustrated in Fig. 1. The free length between the surfaces of the molecules is on the average $L_T = k(V_T^{\frac{1}{3}} - V_0^{\frac{1}{3}})$ where V_T is the molar volume at the temperature $T°$ K.

For associated substances the free length depends on the degree of association as is illustrated in Fig. 2. If the density is constant the free length increases with increasing association. An equation describing this relationship can be obtained in the following way. The association number x is defined as the average number of monomeric molecules clustered together in the liquid. The molar volumes are x times those of the monomeric substance and the free length in the associated liquid is given by

$$L_{poly} = k(x^{\frac{1}{3}} V_T^{\frac{1}{3}} - x^{\frac{1}{3}} V_0^{\frac{1}{3}}) \tag{2}$$

Further, it is convenient to introduce the hypothetical free length L_{mono} which would prevail in the liquid (with unchanged density) if there were no association and the molecules were evenly distributed as is shown in Fig. 2. Thus

$$L_{mono} = k(V_T^{\frac{1}{3}} - V_0^{\frac{1}{3}}) \tag{3}$$

From (2) and (3) the association number can be obtained

$$x = (L_{poly}/L_{mono})^3 \tag{4}$$

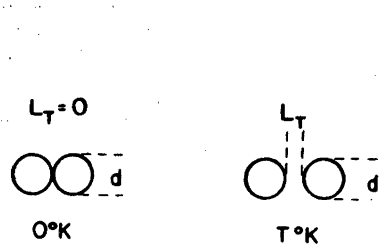

Fig. 1.

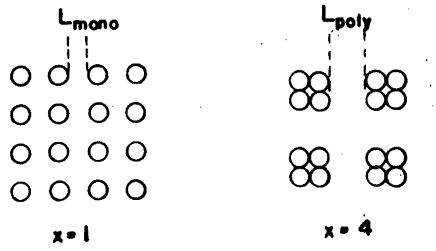

Fig. 2. Illustration of basic principle for calculation of association number x *from equation (4).* L_{poly} *is the actual free length in the associated liquid and* L_{mono} *is a hypothetical free length which would prevail if the molecules were unassociated and evenly distributed.*

This equation is the basic formula for determining association numbers in this work.

Another relationship sometimes useful for determining association numbers in homologous series is

$$x = (d_{poly}/d_{mono})^3 \qquad (5)$$

which is obtained by an argument analogous to that used in obtaining equation (4). The actual average diameter of the associated molecules is d_{poly}, and d_{mono} is the hypothetical diameter which the molecules would have if they were unassociated.

Liquid mixtures can be treated in the same way as pure liquids utilizing free length relations if the molecular size of the two or more components is of the same order of magnitude. The molecular weight of a mixture is $M_{1,2\ldots n} = m_1M_1 + m_2M_2 + \ldots + m_nM_n$ where M_n and m_n are the molecular weight and mole fraction of the compound n.

APPARENT CRITICAL TEMPERATURE

The critical temperature for a liquid system can be obtained graphically from the ratio between the density values at two temperatures [8]. The method is applicable to both pure liquids and liquid mixtures.

For associated systems this method is of special interest for the following reasons. In order to calculate the zero point molar volume, to be used in equation (3), for an associated liquid, its reduced temperature must be known [8]. For that purpose one could not use the observed critical temperature since the critical temperature value is strongly dependent on the size of the molecular aggregates. Since the degree of association changes with temperature the observed critical temperature has no significance for predicting the thermal contraction of the liquid except near the critical point. For determining the reduced temperature at any temperature far from the critical point one should instead use an apparent critical temperature $T_{c,app}$ defined as the critical temperature the liquid would have if the degree of association did not change with increasing temperature. Since the association can be regarded as constant within small temperature ranges, such an apparent critical temperature at any temperature T can be obtained according to the

method described previously [8]. It is necessary to know the densities at two temperatures $T + \Delta T$ and $T - \Delta T$ where ΔT is about 5 to 10°. Apparent critical temperatures have been calculated for some associated liquids (Table 2). In general the apparent critical temperature is larger than the observed critical temperature. This is consistent with the fact that the degree of association is generally larger at lower temperatures.

When the apparent critical temperature is known at a certain temperature the zero point density can be obtained as well as the hypothetical free length L_{mono} at any temperature [8]. The hypothetical diameter d_{mono} can also be calculated.

EMPIRICAL FREE LENGTH RELATIONSHIPS

To arrive at L_{poly} in equation (4) or d_{poly} in equation (5) it is necessary to employ certain empirical relationships.

The adiabatic compressibility β and the surface tension σ can be related to the intermolecular free length [5,6]. If the free length is defined as stated above and the zero point density is calculated according to the method previously described [8] the following relations hold

$$\beta = k_\beta L^{3.4} \tag{6}$$

$$\sigma = k_\sigma L^{-1.8} \tag{7}$$

L represents either L_T in a normal liquid or L_{poly} in an associated liquid. The constants $k\beta$ and $k\sigma$ vary slightly with the temperature (Table 1). Equation (6) makes it possible to determine the free length in any liquid or liquid mixture from its adiabatic compressibility. The compressibility can be accurately obtained from experimental values for the sound velocity and the density [10,5]. Equation (7) makes it possible to determine the free length in a pure liquid from its surface tension.

Table 1. Values for the constants k_β *and* k_σ *in cgs-units at various temperatures.*

Temp °C	0	10	20	25	30	40	50
log k_β	9.770	9.752	9.736	9.728	9.722	9.711	9.702
log k_σ	−13.530	−13.509	−13.490	−13.481	−13.472	−13.456	−13.441

It is of interest to know the accuracy of equations (6) and (7). The average deviation in free lengths, determined as previously [5,6], is 2.1 % for 42 normal organic liquids of various types in the case of equation (6). The corresponding deviation for equation (7) was 2.3 %. No systematic errors occur due to specific elementary compositions or from certain types of chemical constitution (Fig. 3).

The critical temperature value for a liquid depends on the molecular size and the elementary composition. For homologous series the critical temperature has been found to be a linear function of the product of the molecular diameter d and the zero point density ϱ_0 (Fig. 4). In general, the relationship can be written

$$T_c = k_1 \varrho_0 d + l \tag{8}$$

where k_1 and l have constant values for each homologous series. The relation, tested for 25 substances, holds with an average accuracy of better than 1 % in the values for the critical temperature.

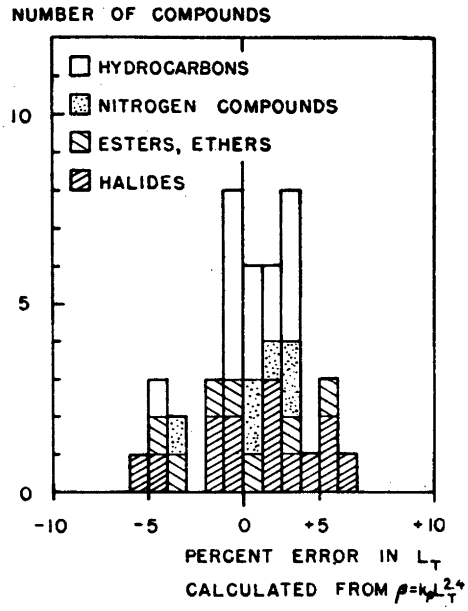

Fig. 3. Error distribution plot of equation (6) tested by 42 normal organic liquids.

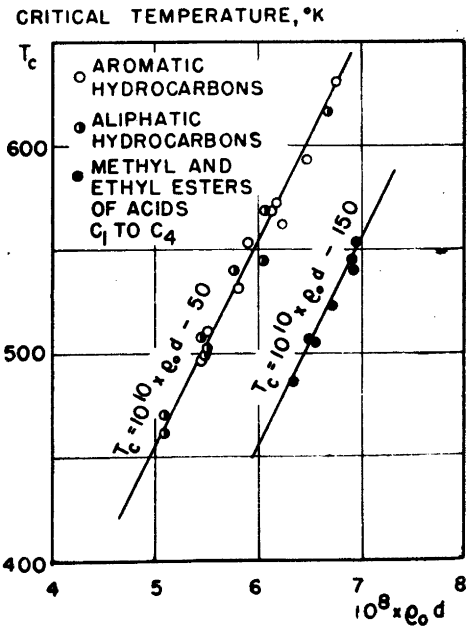

Fig. 4. For homologous series the critical temperature is a linear function of the product of the zero point density σ_0 and the molecular diameter d.

Table 2. Association numbers (x) calculated by various methods.

Compound	Temp. $t°$ C	Measured critical temp.[a] T_c °K	Apparent critical temp. at $t°$[b] $T_{c,app}$ °K	x calcd. from compressibility[c]	x calcd. from surface tension[d]	x calcd. from $T_{c,app}$ Relative accuracy ± 0.1	Selected value for x	Associated molecular weight xM
Methyl alcohol	20	513.3	553	3.53	(3.01)	(3.53)[e]	3.5	112
Ethyl alcohol	0	516.3	593	2.81				
	10		590	2.77	2.76			
	20		586	2.70	2.70	2.77	2.7	124
	30		582	2.62	2.65			
	40		578	2.52	2.59			
	50		574	2.41	2.51			
n-Propyl alcohol	20	536.9	624	2.35	2.33	2.32	2.3	138
iso-Propyl alcohol	20	516.7	598	2.30	2.31	2.24	2.3	138
n-Butyl alcohol	20	560.2	639	1.90	1.94	1.95	2.0	148
iso-Butyl alcohol	20	552.3	636	2.06	2.12	1.96	2.0	148
sec-Butyl alcohol	20		619			1.84	1.8	133
tert-Butyl alcohol	20		569	1.62	1.85	1.64	1.6	119
n-Amyl alcohol	20		670	1.78	1.82	1.78	1.8	159
active Amyl alcohol	20		650			1.69	1.7	150
sec-Amyl alcohol	20		625		1.58	1.56	1.6	141
tert-Amyl alcohol	20		568		1.21	1.30	1.3	115
Diethylcarbinol	20		608		1.39	1.44	1.4	123
n-Hexyl alcohol	20		686	1.60	1.86	1.59	1.6	163
n-Octyl alcohol	20		705	1.29	1.48	(1.29)[e]	1.3	169
Ethylene glycol	20		845	2.44			2.4	149
Glycerol	20		1 030	2.45			2.5	230
Benzyl alcohol	20		749	1.31	1.34		1.3	141
Formic acid	20		608	2.25	1.96		2.2	101
Acetic acid	20	594.8	590	2.12	1.97		2.1	126
Propionic acid	20	610.8	581	1.56	1.49		1.6	119
n-Butyric acid	20	627.9	600	1.39	1.36		1.4	123
n-Valeric acid	20	652.1	624	1.28	1.26		1.3	133
Acetone	20		504	1.18	1.09		1.2	70
Methylethylketone	20		526	1.07			1.1	79
cyclo-Hexanone	20		636	1.48	1.45		1.5	147
Formamide	20		745	2.97	(1.81)		3.0	135
Phenylhydrazine	20		761	1.02	1.20		1.1	119
(Aniline)[f]	20		687	0.95	0.99		1.0	93
(Nitromethane)[f]	20		553	1.09	1.00		1.0	61
(Water)[g]	20	647.2	(2 000)	(225)	(73)	(60) [h]	(60)	(1 080)
	30		(1 480)	(77)	(28)	(30) [h]	(30)	(540)
	40		(1 260)	(39)	(16)	(21) [h]	(20)	(360)
	50		1 120	24	11	16 [h]	15	270
	60		990	14	7.0	13 [h]	13	234
	70		910	9.9	5.1	10 [h]	10	180
	80		850	7.2	4.0	9.1[h]	7	126
	90		820	5.8	3.5	8.4[h]	6	108

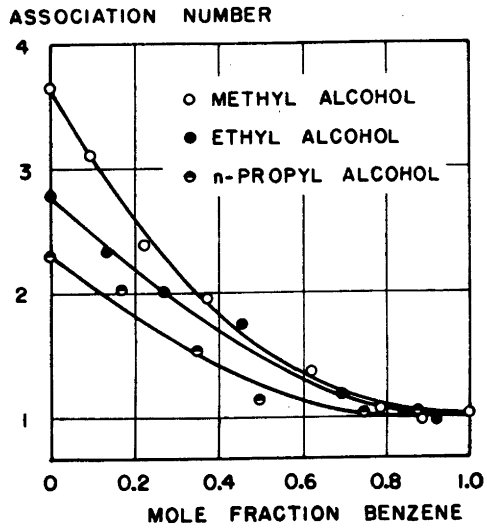

Fig. 5. Association numbers in benzene mixtures with some alcohols.

RESULTS

Utilizing values for L_{poly} determined from compressibility or surface tension data, and L_{mono} from thermal expansion data, association numbers for pure liquids can be calculated from equation (4). Some examples are given in Table 2.

Equation (8) makes it possible in certain cases to calculate relative association numbers from the apparent critical temperatures. If the association numbers are known for two compounds in an homologous series the constants in equation (8) can be evaluated and the associated molecular diameter d_{poly} can be calculated for the other compounds in the series. Since d_{mono} can be obtained from equation (1) association numbers can be calculated from equation (5). Utilizing the association numbers determined for methyl alcohol and octyl alcohol from compressibility data, the constants for aliphatic alcohols were determined to $k_1 = 1.9 \times 10^{10}$ and $l = 698$.

a Values taken from Timmermans [9].
b Calculated from ratio of densities at 15° and 25°, or 0° and 30°, or 15 and 30° C. Accuracy ± 10°.
c Compressibility values taken from Bergmann or Schaaffs [10]. Maximum error in x estimated to ± 0.3 units or ± 15 %.
d Surface tension values taken from Timmermans [9] and Landolt Börnstein Tabellen. Maximum error in x estimated to ± 0.3 units or ± 15 %.
e Chosen value for determining constants in equation (8).
f Non-associated compound included for comparison.
g Lattice associated liquid. Values given are order of magnitude.
h Calculation made under the assumption that water is the first homologue of the alcohols.

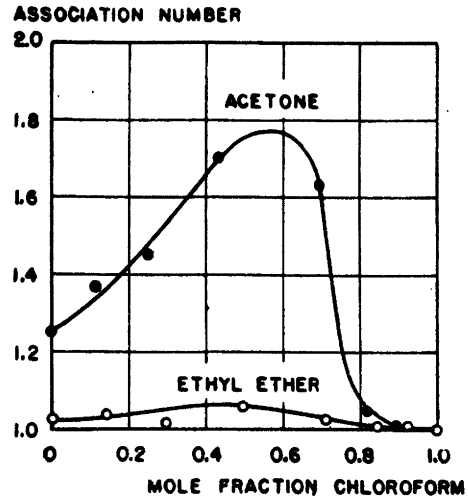

Fig. 6. Association numbers in chloroform mixtures with acetone and diethyl ether.

For liquid mixtures association numbers can be obtained from compressibility data in the same way as used for pure liquids. Ultra sonic velocity, density and thermal expansion were measured for a number of liquid systems with an experimental technique previously described [11]. Some examples of association numbers obtained are given in Figs. 5 and 6. Due to the surface absorption as predicted by Gibbs' adsorption equation [6] formula (7) cannot be used for calculating free lengths in the bulk of liquid mixtures. Therefore, association numbers cannot be obtained in liquid mixtures from surface tension data.

Errors in the association numbers calculated arise from three different sources. In the first place the molecular model used assumes spherical shape which is an oversimplification. This problem was investigated in a previous study [5] and it was found unnecessary to employ any form factors for low molecular weight substances. Secondly, there are uncertainties involved in the determination of the apparent critical temperature and the zero point density [8]. Furthermore, the empirical relationships (6), (7) and (8) have certain inaccuracies as discussed above.

If the errors from different sources are added together in the most unfavourable manner the maximum error for the compressibility and the surface tension method is found to be $\pm$ 0.3 association number unit or $\pm$ 15 % whichever is the larger. The probable error is less than $\pm$ 0.2 association number unit. The apparent critical temperature method for calculating relative association numbers from equation (5) and (8) in homologous series, holds with an accuracy better than $\pm$ 0.1 association number unit.

DISCUSSION

Among associated systems one can distinguish between such cases where a true cluster association occurs, as is the case for certain organic liquids, and such cases where a quasicrystalline order prevails throughout the whole liquid, as for water. In the former case the calculation of association numbers from free length relationships is straightforward. In the latter case, which will be referred to as lattice associated systems, the calculation of association numbers does not have the same clear physical significance. The distinction between these two cases, however, is more a matter of degree than of type.

The free length method for determining association numbers seems to offer a possibility for distinguishing between the two types of association. The surface molecule in a molecular lattice does not have the same freedom to associate with its neighbouring molecules as does a bulk molecule. Therefore, it is to be expected for a lattice associated liquid that the association numbers determined from the surface tension are lower than those calculated from the compressibility. As is seen from Table 2 this is the case for water. Methyl alcohol and formamide are also slightly lattice associated which is possible in view of their chemical constitution. When the association number obtained from compressibility data agrees within the limits of experimental error with that calculated from surface tension data, the liquid is cluster associated.

For the cluster associated liquids the associated molecular weights in Table 2 lies between 70 and 230. The values are of a reasonable order of magnitude since most organic liquids have molecular weights in that range [4]. Non-associated, saturated, organic substances that contain oxygen or nitrogen generally are in the gaseous state at room temperature if their molecular weight is below approximately 65. They are generally in the solid state if the weight is larger than 250 to 300.

It is of interest to observe that for the series of homologues the association numbers decrease with increasing length of the chain. This is to be expected from the increasing paraffin character of the substances. For isomeric compounds such as butyl alcohols or amyl alcohols the association numbers decrease with increased branching of the chain. This is probably due to a steric hindrance of the association. For propionic acid and the higher carboxylic acids the apparent critical temperatures are lower, by about 5 %, than the observed critical temperatures. This anomalous behavior, if significant, would indicate that the degree of association is slightly larger at higher temperatures than at lower temperatures. Another experimental observation consistent with this conclusion is that the dielectric constant increases with increasing temperature for the carboxylic acids contrary to most other substances [12].

The results obtained for the liquid mixtures constitute a support for the correctness of the free length method for calculating association numbers. Other methods of investigation [3] have shown that the association of the alcohols is hindered by the presence of benzene. The results obtained here are in accordance with these observations. In the case of acetone and chloroform a complex formation [3], known from other methods, causes the association number to have a maximum at about 0.5 mole fraction. The same is true for ether-chloroform though the complex formation apparently is less pronounced.

This is probably because the oxygen atom in ether, due to steric hindrance, is less accessible for hydrogen bonding than the oxygen in acetone. The fact that pure ether is unassociated whereas acetone is slightly associated offers further evidence that this is a probable explanation for the differences observed in the two kinds of mixtures.

It should be observed that the free length method gives no information about the type of association taking place in a mixture. It only tells on the average if the mean cluster size is smaller, equal to or lager than that expected if no change of association occurs on mixing.

As stated above the concept of free length has no significance when the molecules are bound together with highly directional forces similar to those in the solid state. However, with rising temperature the lattice order is reduced and the system gets more like that of an ordinary liquid. Above some temperature, therefore, it may be possible to treat the liquid as a cluster associated system. In Table 2 the results for water have been tabulated for the temperature range 20° to 90° C. Above 50° the results from the compressibility method and the apparent critical temperature method generally agree within a factor of about 1.5. Furthermore, the associated molecular weights obtained above 50° are of a reasonable order of magnitude in view of the physical properties of water. Therefore, it appears as if the free length method could be used above that temperature to determine the order of magnitude of the association of water. At temperatures lower than 50° C quantitative results cannot be obtained for pure water or dilute aqueous solutions. Since the association in water generally is strongly reduced by the presence of other molecules, the temperature limit for qualitative studies is lowered with increasing concentration of the non-aqueous component.

The compressibility of water has a minimum at about 60° C [10]. The decrease between 0 and 60° is the result of a breakdown of the association so that the free length decreases in spite of the increase in specific volume. The same explanation applies to the minimum observed when the compressibility is studied as a function of increasing concentration of a liquid in mixture with water [11].

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Short Communications

Correlation between Steric Configuration and Growth-regulating Activity of α-(3-Chloro-2-naphthoxy)propionic Acid

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The resolution of α-(3-chloro-2-naphthoxy)propionic acid (I) into optical antipodes has recently been reported by Pope and Woodcock [1]. According to these authors the (+)-form of the acid (I) is a highly active plant-growth substance while the (−)-form is inactive. Several other optically active plant-growth substances have been investigated by Matell [2-4] and by Åberg [5]. The establishment of the configuration of these compounds led to the conclusion that D-configuration is a necessary condition for auxin activity while L-configuration is associated with anti-auxin activity or inactivity. According to this rule the (+)-form of the acid (I) should belong to the D-series. This conclusion has now been verified by connecting the 3-chloro acid (I) sterically to α-(1-chloro-2-naphthoxy)propionic acid (II). This could be done by means of the quasi-racemate method [6-8].

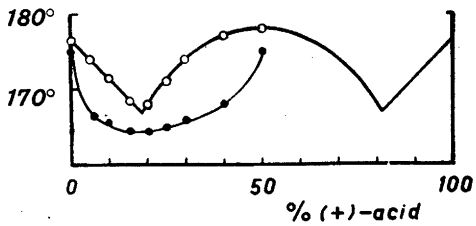
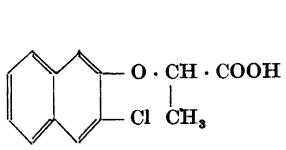
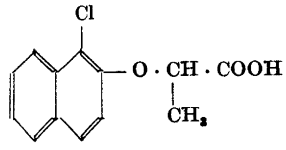


Fig. 1. Melting point diagram of (+) - and (−)-α-(3-chloro-2-naphthoxy)propionic acid.

The racemic form of the acid (I) is a true racemate as can be seen from Fig. 1. The acid (II) has a still greater tendency to racemate formation as the middle branch of its melting point curve extends over 80 % of the concentration interval and the racemate melts 10° higher than the antipodes [9]. Thus the conditions for the formation of a quasi-racemate between the acids (I) and (II) are favourable not only because of their structural similarity. The melting point diagram of the acids (I) and (II) with opposite directions of rotation (Fig. 2) shows the existence of a molecular compound in the ratio 1:1, which is evidently a quasi-racemate as no molecular compound is formed between the acids with the same direction of rotation (Fig. 3). The configuration of the 1-chloro isomer (II) is known; the (+)-form belongs to the D-series [9,10]. By definition the components of a quasi-racemate have opposite configurations and thus the (+)-form of the 3-chloro isomer (I) has D-configuration. It is represented by the stereoformula (III).



(I)



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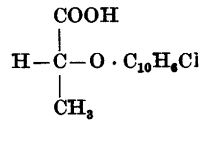

(III)

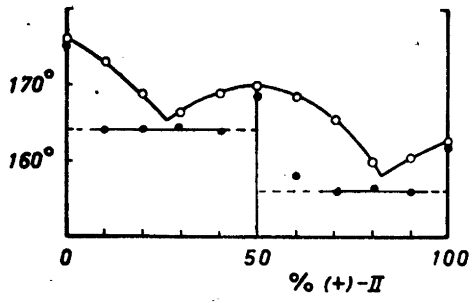

Fig. 2. Melting point diagram of (—)-α-(3-chloro-2-naphthoxy)propionic acid (I) and (+)-α-(1-chloro-2-naphthoxy)propionic acid (II).

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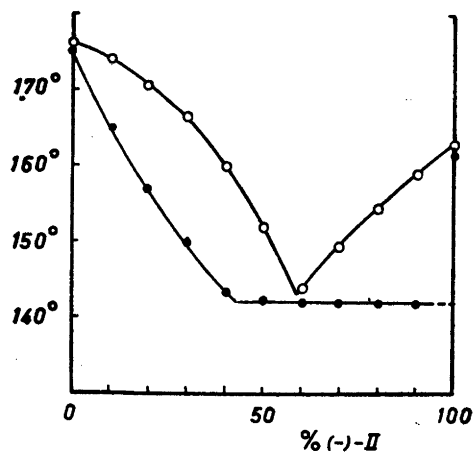

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On the Formation of Boric Acid -Mannitol Complexes

P. J. ANTIKAINEN

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The formation of boric acid-mannitol complexes has been the object of many studies, but only a few quantitative investigations of the reaction in question have been published. The author of this paper has previously studied [1] the influence of mannitol on the ionization of boric acid in aqueous solutions of some alkali salts and shown that the apparent ionization constant K^* of boric acid in dilute boric acid solutions can be expressed in the form:

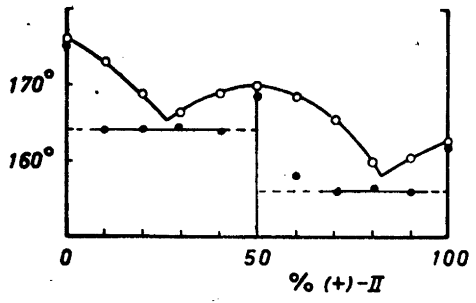
$$K^* = K \cdot C^n, \qquad (1)$$

where C is the stoichiometric concentration of mannitol, n the average number of mannitol molecules which combine with one molecule of boric acid and K the equilibrium constant for the reaction:

$$HB + n\ M \rightleftharpoons H^+ + BM_n^-, \qquad (2)$$

where HB and M designate boric acid and mannitol, respectively, and BM_n^- the anion of the acid formed.

The values of the complex formation constant K obtained by previous workers [2-8] show a considerable variation with varying concentration of mannitol. Most probably one reason for this variation lies in the



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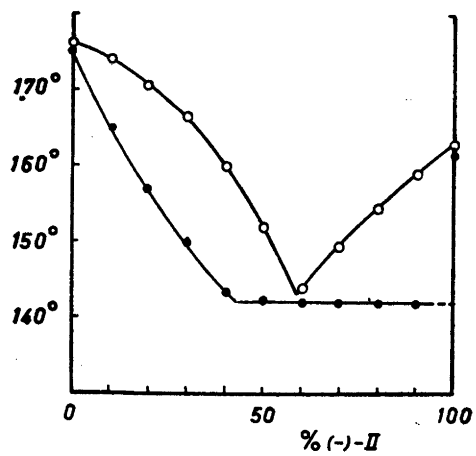

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$$K^* = K \cdot C^n, \qquad (1)$$

where C is the stoichiometric concentration of mannitol, n the average number of mannitol molecules which combine with one molecule of boric acid and K the equilibrium constant for the reaction:

$$HB + n\ M \rightleftharpoons H^+ + BM_n^-, \qquad (2)$$

where HB and M designate boric acid and mannitol, respectively, and BM_n^- the anion of the acid formed.

The values of the complex formation constant K obtained by previous workers [2-8] show a considerable variation with varying concentration of mannitol. Most probably one reason for this variation lies in the

fact that the value 2 generally has been used for the constant n, although recent findings indicate that this constant should have a value of about 1.81 [1]. Using this value for the constant n the complex formation constant K can be calculated according to the author [1] with an average accuracy of 0.01 pK-units at different concentrations of mannitol.

The above-mentioned deviation of the term n from the whole number can be explained by assuming that besides the main complex HBM_2 small quantities of the complex HBM are formed simultaneously. Thus equation (1) can be written in the form:

$$K^* = K_1 \cdot C^2 + K_2 \cdot C, \qquad (3)$$

where K_1 and K_2 are the equilibrium constants for the reactions:

$$HB + 2\ M \rightleftharpoons H^+ + BM_2^- \qquad (4)$$

and

$$HB + M \rightleftharpoons H^+ + BM^-$$

respectively. For the determination of these constants by means of equation (3) it is necessary to know the values for the apparent ionization constants at at least two different concentrations of mannitol. These values can be obtained from equation (1) using the already known values [1] for the constants K and n. Results of calculations are given in Table 1, where C' and C'' designate the two different concentrations of mannitol and K' and K'' the corresponding apparent ionization constants of boric acid. The concentrations C' and C'' have been selected so that the differences between them are as great as possible insuring that the calculations are correct. Furthermore these concentrations are so high that equation (1) is in good agreement with theory [1].

As can be seen from Table 1 the values for the complex formation constant K_2 can be calculated with an average accuracy of 0.02—0.05 pK-units using the above-mentioned method. Thus it is apparent that a complex of the type HBM also exists in solutions of boric acid and mannitol confirming previous statements [1,3,4].

For the constant pK_2 the values 5.65 and 6.70 were obtained by Böeseken [3] and Deutsch [8], respectively. The corresponding thermodynamic constant has now been calculated and found to be 5.218 ± 0.05 at 25° C and thus for the thermodynamic constant K_1 the value 4.357 ± 0.02 can be calculated by means of equation (3).

Table 1. Determination of the complex formation constants of mannitoboric acid in aqueous potassium chloride solutions at 25° C.

C'	C''	$K' \cdot 10^5$	$K'' \cdot 10^5$	pK_2	Average
0.3	1.0	0.564	4.975	5.256	
0.3	0.8	0.564	3.320	5.287	$\sqrt{I} = 0.0$
0.5	1.0	1.421	4.975	5.153	pK_1 = 4.359
0.5	0.8	1.421	3.320	5.182	pK_2 = 5.218
0.3	1.0	0.837	7.500	5.113	
0.3	0.8	0.837	5.008	5.106	$\sqrt{I} = 0.316$
0.5	1.0	2.100	7.500	5.046	pK_1 = 4.181
0.5	0.8	2.100	5.008	5.115	pK_2 = 5.095
0.3	1.0	0.904	8.020	5.062	
0.3	0.8	0.904	5.340	5.093	$\sqrt{I} = 0.632$
0.5	1.0	2.282	8.020	4.956	pK_1 = 4.149
0.5	0.8	2.282	5.340	4.978	pK_2 = 5.022
0.3	1.0	0.655	5.803	5.198	
0.3	0.8	0.655	3.870	5.297	$\sqrt{I} = 1.412$
0.5	1.0	1.652	5.803	5.097	pK_1 = 4.289
0.5	0.8	1.652	3.870	5.137	pK_2 = 5.128

The values for the complex formation constants at different ionic strenghts agree with Debye-Hückel's equation from which the values $a = 2.375$ and 2.115 and B = 0.133 and 0.108 for the constants K_1 and K_2 can be calculated.

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Enzymic Synthesis of Ureidosuccinic Acid from Citrulline *via* Compound X and Carbamyl Phosphate

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The first step in the enzymic synthesis of citrulline involves the formation of an intermediate (compound X) which contains ammonia and CO_2 in an activated form [1]. Citrulline is formed by condensation of the intermediate with ornithine. USA ** arises when the same intermediate condenses with aspartic acid [2].

Grisolia and Cohen [1] showed that the presence of ATP, Mg^{++} and an N-substituted derivative of glutamic acid, *e. g.* carbamyl glutamate or acetyl glutamate, was required for the formation of compound X. They proposed originally that the intermediate contained ammonia, CO_2 and phosphate bound to an N-substituted glutamic acid in an unknown way. Recently Jones *et al.*[3], however, made the very important demonstration that carbamyl phosphate was formed in an extract of *Streptococcus faecalis* and that CAP ** in liver preparations readily formed citrulline in the presence of ornithine. Indirect evidence indicated that USA also could be formed from CAP and aspartic acid. The possibility was therefore considered that CAP and compound X were identical. Further strong evidence for this concept was presented by Marshall *et al.*[4] On the other hand experiments by Grisolia *et al.*[5] indicated that CAP was not identical with the compound X prepared by these authors.

We have recently demonstrated the formation of USA from citrulline-ureido-^{14}C and aspartic acid in the presence of acetylglutamate, ATP and Mg^{++} in an extract from rat liver mitochondria [6]. The requirement of the reaction for acetyl glutamate implicated the intermediate formation of compound X. We have now studied this question by investigating the formation of radioactive compounds during incubation of citrulline-ureido-^{14}C with acetyl glutamate, ATP and Mg^{++} in mitochondrial extracts.

After deproteinization and addition of carrier CAP the solution was made slightly alkaline (phenolphthalein) and precipitated with barium acetate. After centrifugation two volumes of alcohol were added to the supernatant and the precipitated barium salts were collected and analyzed by paper electrophoresis. The paper strips were scanned for radioactivity (Fig. 1). The presence of acetyl glutamate during incubation resulted in the appearance of two radioactive bands, one of which coincided with CAP. Both bands also depended on the presence of ATP. When the paper strips were sprayed with 0.05 *N* HCl and dried in a stream of warm air the radioactivity in the two fast moving bands disappeared. This type of behaviour would be expected from acid labile compounds such as CAP or CAP bound to acetyl glutamate.

These two radioactive bands were also found in experiments where compound X was formed from NH_3, $NaH^{14}CO_3$, ATP, Mg^{++} and acetyl glutamate (*cf.*[1]). Again the bands depended on addition of acetyl glutamate, and spraying with acid removed the radioactivity. In these experiments no carrier CAP was added before barium precipitation.

When compound X was formed from radioactive carbamyl glutamate, $NaHCO_3$, NH_3, ATP and Mg^{++}, it was found that only the fastest moving band was radioactive and that the radioactivity did not disappear on spraying with acid.

Fractionation of the radioactive compounds was also carried out by ion exchange chromatography on Dowex-2-formate columns. In this case the deproteinized solution was put directly on the column after neutralization to pH 8 without addition of carrier CAP. Gradient elution with ammonium formate, pH 8.0, was performed. Again two radioactive peaks were observed, whose presence depended on the inclusion of acetyl glutamate during incubation. The first of these peaks corresponded in position to CAP, while the second was retained more strongly by the column. Both peaks lost radioactivity after acidification and warming. When ornithine and a mitochondrial extract were added to the material from each peak formation of citrulline could be demonstrated. The second peak was radioactive in experiments with carbamyl glutamate-^{14}C.

The evidence presented here indicates that from both $NaH^{14}CO_3$ or citrulline-ureido-^{14}C two radioactive compounds

* Fellow of U.S. Public Health Service.

** The following abbreviations are used in this paper: ATP, adenosine triphosphate; USA, ureidosuccinic acid; CAP, carbamyl phosphate.

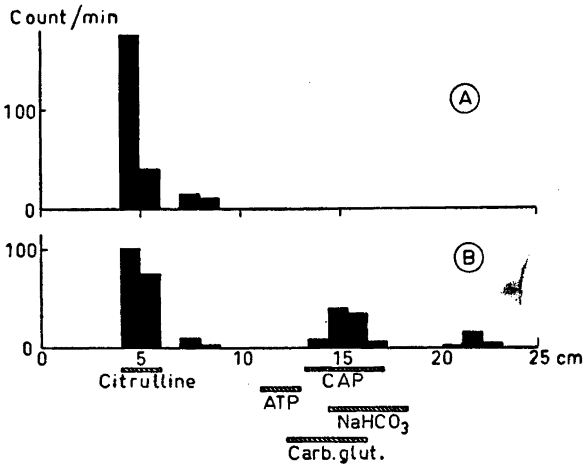


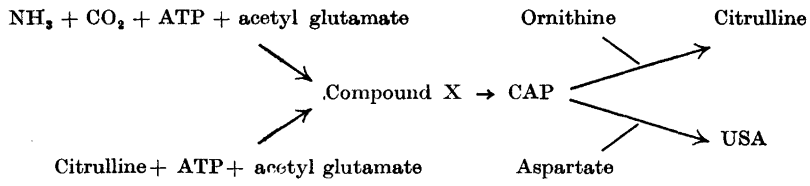
Fig. 1. Five ml of an extract of rat liver mitochondria (7 mg protein nitrogen) were incubated at 37° for 20 min. with 20 µmoles of citrullineureido-^{14}C (90 000 ct/min/µmole), 40 µmoles of acetyl glutamate, 80 µmoles of ATP, 200 µmoles of $MgSO_4$ and 1 000 µmoles of glycyl-glycin buffer, pH 8.0, in a final volume of 9 ml. An alcohol insoluble barium fraction was prepared as described in the text. After decomposition with Na_2SO_4 an aliquot was subjected to paper electrophoresis with saturated sodium tetraborate at 0° (12 volts/cm, 4 hours). After drying at room temperature the paper strips were directly scanned for radioactivity.

A = acetyl glutamate excluded from incubation mixture.
B = complete substrate.
The figure gives also the positions of different compounds on the paper strips as determined in separate parallel experiments.
The distance was measured from the starting line towards the anode.

were formed in the presence of acetyl glutamate. The more acid compound was also formed from carbamyl glutamate-^{14}C. Both compounds could form citrulline in the presence of ornithine. One of the compounds behaved like synthetic CAP during paper electrophoresis, ion exchange chromatography and also paper chromatography. The other more acid compound seemed to contain CAP bound to acetyl glutamate (or carbamyl glutamate) and thus would be identical in structure with the originally formulated compound X. This second compound amounted in our experiments to between 5 and 20 % of the CAP at the end of the incubation. It is likely, however, that different proportions are obtained under other experimental conditions.

Since acetyl glutamate was necessary for the formation of CAP it seems reasonable to assume that compound X (= CAP bound to acetyl glutamate) is the first active intermediate formed in citrulline and USA synthesis and that CAP arises from it. The following tentative reaction sequence is put forward: See below.

The intermediate position of CAP in USA synthesis was established by enzyme



experiments where $^{15}NH_4Cl$, ATP, $MgCl_2$, acetyl glutamate and aspartate-^{14}C were substrates. The influence of increasing amounts of ^{13}CAP on the isotopic pattern of the formed USA was studied. The results were in full agreement with the scheme, since the ^{15}N-content of USA sharply decreased with increasing amounts of CAP, while the ^{13}C-content increased and finally reached the same level as the ^{14}C-content. Similar results were obtained with labeled citrulline in place of ammonia.

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The Preparation of 2,6-Dichlorophenylacetic Acid

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Ortho halogen substituted benzoic, phenylacetic and phenoxyacetic acids have in recent years been frequently tested as to their auxin activity, among others by Thimann [1]. The activity of a benzoic acid is greatly increased by di-substitution of halogen atoms in 2,6-position, while the same substitution in a phenoxyacetic acid completely destroys the activity.

The 2,4,6-trichlorophenylacetic acid was recently prepared by Jönsson [2], but the 2,6-dichlorophenylacetic acid is not earlier described. By a method related by Fierz-David and Blangey [3] 2,6-dichlorotoluene was prepared. This compound was chlorinated to benzyl chloride which was treated with potassium cyanide to benzyl cyanide and hydrolysed to phenylacetic acid as Staedel [4] has described.

For the sake of control a sample of the 2,6-dichlorotoluene was oxidized, yielding pure 2,6-dichlorobenzoic acid with m. p. 140–141°.

Experimental: 2,6-Dichlorobenzyl chloride. 16.1 g of 2,6-dichlorotoluene was chlorinated with dry chlorine at 180° in ultra-violet rays until an increase of the weight of 3.5 g had been obtained. The product was fractionated *in vacuo* yielding 12 g (61 %) of colourless oil, boiling at 114—119°/13 mm, melting point 11—12°.

2,6-Dichlorobenzyl cyanide. 6.5 g of benzyl chloride was refluxed 5 hours with 2.7 g of potassium cyanide and 30 ml alcohol. The alcohol was distilled off from the solid.

2,6-Dichlorophenylacetic acid. The before mentioned cyanide was refluxed with 1 *N* sodium hydroxide overnight. After extraction with ether the mixture was acidified with 2 *N* hydrochloric acid. The dichlorophenylacetic acid was obtained as a colourless solid (4.0 g; 60 %) which was repeatedly crystallised from aqueous ethanol. Melting point 158—159°. (Found: Equiv. wt. 205.4; Cl 34.41 %. Calc. for $C_8H_6O_2Cl_2$: Equiv. wt. 205.0; Cl 34.59 %).

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The Structure of Tellurium Dibenzenethiosulphonate

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This compound [1], $Te(S_2O_2C_6H_5)_2$, is an analogue of tellurium dimethanethiosulphonate, $Te(S_2O_2CH_3)_2$, and of salts of telluropentathionic acid, $Te(S_2O_2OH)_2$. The crystal structure has been worked out and found to be closely analogous to that of

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tellurium dimethanethiosulphonate [2] and of ammonium telluropentathionate [3].

The crystals of tellurium dibenzenethiosulphonate are orthorhombic bipyramidal, with the axial engths [1]: $a = 14.46$ Å, $b = 11.18$ Å, $c = 10.48$ Å. There are four molecules per unit cell, and the space group is D_{2h}^{14}-*Pbcn*. The tellurium atom is located on a twofold axis of symmetry.

The intensities of the *h*0*l* and *hk*0 reflections were estimated visually from zero layer Weissenberg photographs taken with Cu*K* radiation, and the structure was solved through Patterson and Fourier projections along the *b* and *c* axes. The *h*0*l* Fourier map, with lines drawn to show the outline of a molecule, is reproduced in Fig. 1.

The coordinates of the atoms of the asymmetric unit, in fractions of corresponding cell edges and referring to a centre of symmetry as an origin, are as follows.

With the atomic scattering curves of the *International Tables*, and a temperature factor of $B = 2.4$ Å^2, these coordinates give reliability factors of $R = 0.16$ and 0.17, respectively, for the *h*0*l* and *hk*0 reflections.

	x	*y*	*z*
Te	0	0.043	0.250
S_2	—0.068	0.186	0.395
S_1	0.041	0.233	0.514
O_1	—0.006	0.316	0.598
O_2	0.083	0.139	0.566
C_1	0.117	0.312	0.413
C_2	0.207	0.271	0.396
C_3	0.268	0.316	0.316
C_4	0.238	0.435	0.253
C_5	0.148	0.476	0.270
C_6	0.087	0.414	0.349

The S—S—Te—S—S chain is unbranched and has a *trans* [4] configuration. The bond lengths and angles are, Te—S_2 = 2.41 ± 0.03 Å, S_2—S_1 = 2.08 ± 0.03 Å, ∠ S_2TeS_2' = 97 ± 2°, ∠ TeS_2S_1 = 104 ± 2°, and S_1S_2Te/S_2TeS_2' (dihedral angle) = 79°. These values are in fair agreement with earlier data [2,3] for such chains. The benzenesulphonyl groups have, likewise, normal dimensions.

Details of the work will be published later.

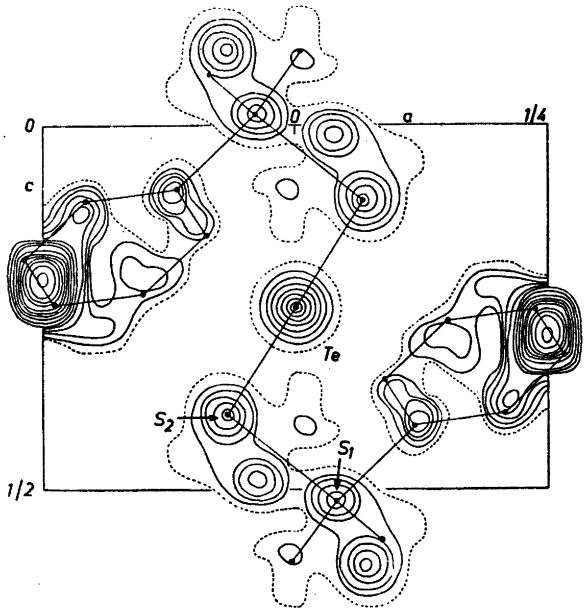

Fig. 1. Electron density projection of $Te(S_2O_2C_6H_5)_2$ *along the* b *axis. Contour lines are at intervals of 1 e·Å^{-2} for carbon atoms, 5 e·Å^{-2} for sulphur and oxygen atoms, and 15 e·Å^{-2} for the tellurium atom, starting with 6, 10, and 15 e·Å^{-2}, respectively. The 5-electron line is dashed.*

1. Foss, O. *Acta Chem. Scand.* **6** (1952) 521.
2. Foss, O. and Vihovde, E. H. *Acta Chem. Scand.* **8** (1954) 1032.
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4. Foss, O. *Acta Chem. Scand.* **7** (1953) 1221.

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The Structure of Tellurium Di-*p*-toluenethiosulphonate

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In a preceding note [1], the crystal structure of tellurium dibenzenethiosulphonate, $Te(S_2O_2C_6H_5)_2$, was described. The crystals are orthorhombic, with a four-molecule unit cell based on the space group $D_{2h}^{14}-Pbcn$, and $a = 14.46$ Å, $b = 11.18$ Å, $c = 10.48$ Å. A twofold axis of symmetry, parallel to the b axis, passes through the tellurium atom at $x = 0$, $z = \frac{1}{4}$. In the projection along the c axis, of symmetry cmm, the tellurium atom lies in an apparent mirror plane.

The six analogous compounds, sulphur [2] and selenium [3] dibenzenethiosulphonate, and sulphur [2], selenium and tellurium di-*p*-toluenethiosulphonate and triselenium di-*p*-toluenesulphinate [3], crystallize in the tetragonal space group, $D_4^4-P4_12_12$ (or the enantiomorph) with four molecules per unit cell. A twofold axis of molecular symmetry is required, as in orthorhombic tellurium dibenzenethiosulphonate. The crystal structure of tetragonal tellurium di-*p*-toluenethiosulphonate, $Te(S_2O_2C_6H_4CH_3)_2$, has been solved by means of reference to the known structure of the benzene analogue, as follows.

In the centered setting, $C4_122_1$, the twofold rotation axes of the tetragonal unit cell of tellurium di-*p*-toluenethiosulphonate become parallel to the a and b axes. The axial lengths then are: $a = 10.93$ Å, $c = 29.88$ Å. These axes, and the centered setting instead of the primitive one, are referred to in the following. It is seen that the tetragonal a axis is of about the same length as the b and c axes of orthorhombic tellurium dibenzenethiosulphonate, *viz.*, 11.18 Å and 10.48 Å, respectively, while the tetragonal c axis is a little longer than twice the orthorhombic a axis, 14.46 Å.

In the projection of $C4_122_1$ along the b axis, the symmetry corresponds to that of the plane group, pgm, twice along the a axis. Considering the projected area, $a/2 \times c$, the eight general positions may be described as made up of two fourfold sets, both of symmetry pgm, with the coordinates, x, z and y, $\frac{1}{4} + z$, respectively, for the first of the four positions of each set. The corresponding coordinates of the special positions on the twofold rotation axes are, 0,0 and y, $\frac{1}{4}$. A comparison with the orthorhombic space group, $Pbcn$, shows that a molecule, belonging to the set, x, z, in the b axis projection of $C4_122_1$ corresponds to a molecule in the b axis projection of $Pbcn$, and one belonging to the set, y, $\frac{1}{4} + z$, corresponds to one in the c axis projection of $Pbcn$. The similarities of tetragonal and orthorhombic cell dimensions made it likely that the projected shapes of thus corresponding molecules were the same, or nearly the same, in the two space groups.

The intensities of the $h0l$ reflections of tellurium di-*p*-toluenethiosulphonate were estimated visually from Weissenberg photographs taken with CuK radiation. A Patterson synthesis based on these data gave the y coordinate of tellurium, and a Fourier synthesis was performed, using the strongest reflections and signs obtained from the tellurium contributions alone. Peaks appeared in the Fourier map at or close to the expected sulphur positions, but the oxygen and carbon positions were uncertain, also after a second Fourier synthesis based on signs from the tellurium and the sulphur contributions. Using the tellurium positions as reference points, and corresponding axes of the two crystals coinciding or parallel, the sulphur, oxygen and carbon atoms of the asymmetric units of the two fourfold sets discussed above were then placed exactly as found in the b and c axis projections, respectively, of orthorhombic tellurium dibenzenethiosulphonate, with *p*-methyl carbons added. This was done by placing the tetragonal Fourier map, drawn on transparent paper, on the orthorhombic maps, of the same scale, and marking the positions. The subsequent structure factor calculation, with appropriate temperature and scale factors, lead to a reliability factor of $R = 0.33$. The most notable effect of the inclusion of the oxygen and carbon contributions was to change the sign of the next to strongest

experiments where $^{15}NH_4Cl$, ATP, $MgCl_2$, acetyl glutamate and aspartate-^{14}C were substrates. The influence of increasing amounts of ^{13}CAP on the isotopic pattern of the formed USA was studied. The results were in full agreement with the scheme, since the ^{15}N-content of USA sharply decreased with increasing amounts of CAP, while the ^{13}C-content increased and finally reached the same level as the ^{14}C-content. Similar results were obtained with labeled citrulline in place of ammonia.

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The Preparation of 2,6-Dichlorophenylacetic Acid

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Ortho halogen substituted benzoic, phenylacetic and phenoxyacetic acids have in recent years been frequently tested as to their auxin activity, among others by Thimann [1]. The activity of a benzoic acid is greatly increased by di-substitution of halogen atoms in 2,6-position, while the same substitution in a phenoxyacetic acid completely destroys the activity.

The 2,4,6-trichlorophenylacetic acid was recently prepared by Jönsson [2], but the 2,6-dichlorophenylacetic acid is not earlier described. By a method related by Fierz-David and Blangey [3] 2,6-dichlorotoluene was prepared. This compound was chlorinated to benzyl chloride which was treated with potassium cyanide to benzyl cyanide and hydrolysed to phenylacetic acid as Staedel [4] has described.

For the sake of control a sample of the 2,6-dichlorotoluene was oxidized, yielding pure 2,6-dichlorobenzoic acid with m. p. 140–141°.

Experimental: 2,6-Dichlorobenzyl chloride. 16.1 g of 2,6-dichlorotoluene was chlorinated with dry chlorine at 180° in ultra-violet rays until an increase of the weight of 3.5 g had been obtained. The product was fractionated *in vacuo* yielding 12 g (61 %) of colourless oil, boiling at 114—119°/13 mm, melting point 11—12°.

2,6-Dichlorobenzyl cyanide. 6.5 g of benzyl chloride was refluxed 5 hours with 2.7 g of potassium cyanide and 30 ml alcohol. The alcohol was distilled off from the solid.

2,6-Dichlorophenylacetic acid. The before mentioned cyanide was refluxed with 1 *N* sodium hydroxide overnight. After extraction with ether the mixture was acidified with 2 *N* hydrochloric acid. The dichlorophenylacetic acid was obtained as a colourless solid (4.0 g; 60 %) which was repeatedly crystallised from aqueous ethanol. Melting point 158—159°. (Found: Equiv. wt. 205.4; Cl 34.41 %. Calc. for $C_8H_6O_2Cl_2$: Equiv. wt. 205.0; Cl 34.59 %).

The author whishes to express his thanks to Professor Arne Fredga for valuable discussions.

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The Structure of Tellurium Dibenzenethiosulphonate

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This compound [1], $Te(S_2O_2C_6H_5)_2$, is an analogue of tellurium dimethanethiosulphonate, $Te(S_2O_2CH_3)_2$, and of salts of telluropentathionic acid, $Te(S_2O_2OH)_2$. The crystal structure has been worked out and found to be closely analogous to that of

1. Foss, O. *Acta Chem. Scand.* **6** (1952) 521.
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The Structure of Tellurium Di-*p*-toluenethiosulphonate

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In a preceding note [1], the crystal structure of tellurium dibenzenethiosulphonate, $Te(S_2O_2C_6H_5)_2$, was described. The crystals are orthorhombic, with a four-molecule unit cell based on the space group $D_{2h}^{14}-Pbcn$, and $a = 14.46$ Å, $b = 11.18$ Å, $c = 10.48$ Å. A twofold axis of symmetry, parallel to the b axis, passes through the tellurium atom at $x = 0$, $z = \frac{1}{4}$. In the projection along the c axis, of symmetry cmm, the tellurium atom lies in an apparent mirror plane.

The six analogous compounds, sulphur [2] and selenium [3] dibenzenethiosulphonate, and sulphur [2], selenium and tellurium di-*p*-toluenethiosulphonate and triselenium di-*p*-toluenesulphinate [3], crystallize in the tetragonal space group, $D_4^4-P4_12_12$ (or the enantiomorph) with four molecules per unit cell. A twofold axis of molecular symmetry is required, as in orthorhombic tellurium dibenzenethiosulphonate. The crystal structure of tetragonal tellurium di-*p*-toluenethiosulphonate, $Te(S_2O_2C_6H_4CH_3)_2$, has been solved by means of reference to the known structure of the benzene analogue, as follows.

In the centered setting, $C4_122_1$, the twofold rotation axes of the tetragonal unit cell of tellurium di-*p*-toluenethiosulphonate become parallel to the a and b axes. The axial lengths then are: $a = 10.93$ Å, $c = 29.88$ Å. These axes, and the centered setting instead of the primitive one, are referred to in the following. It is seen that the tetragonal a axis is of about the same length as the b and c axes of orthorhombic tellurium dibenzenethiosulphonate, *viz.*, 11.18 Å and 10.48 Å, respectively, while the tetragonal c axis is a little longer than twice the orthorhombic a axis, 14.46 Å.

In the projection of $C4_122_1$ along the b axis, the symmetry corresponds to that of the plane group, pgm, twice along the a axis. Considering the projected area, $a/2 \times c$, the eight general positions may be described as made up of two fourfold sets, both of symmetry pgm, with the coordinates, x, z and y, $\frac{1}{4} + z$, respectively, for the first of the four positions of each set. The corresponding coordinates of the special positions on the twofold rotation axes are, 0,0 and y, $\frac{1}{4}$. A comparison with the orthorhombic space group, $Pbcn$, shows that a molecule, belonging to the set, x, z, in the b axis projection of $C4_122_1$ corresponds to a molecule in the b axis projection of $Pbcn$, and one belonging to the set, y, $\frac{1}{4} + z$, corresponds to one in the c axis projection of $Pbcn$. The similarities of tetragonal and orthorhombic cell dimensions made it likely that the projected shapes of thus corresponding molecules were the same, or nearly the same, in the two space groups.

The intensities of the $h0l$ reflections of tellurium di-*p*-toluenethiosulphonate were estimated visually from Weissenberg photographs taken with CuK radiation. A Patterson synthesis based on these data gave the y coordinate of tellurium, and a Fourier synthesis was performed, using the strongest reflections and signs obtained from the tellurium contributions alone. Peaks appeared in the Fourier map at or close to the expected sulphur positions, but the oxygen and carbon positions were uncertain, also after a second Fourier synthesis based on signs from the tellurium and the sulphur contributions. Using the tellurium positions as reference points, and corresponding axes of the two crystals coinciding or parallel, the sulphur, oxygen and carbon atoms of the asymmetric units of the two fourfold sets discussed above were then placed exactly as found in the b and c axis projections, respectively, of orthorhombic tellurium dibenzenethiosulphonate, with *p*-methyl carbons added. This was done by placing the tetragonal Fourier map, drawn on transparent paper, on the orthorhombic maps, of the same scale, and marking the positions. The subsequent structure factor calculation, with appropriate temperature and scale factors, lead to a reliability factor of $R = 0.33$. The most notable effect of the inclusion of the oxygen and carbon contributions was to change the sign of the next to strongest

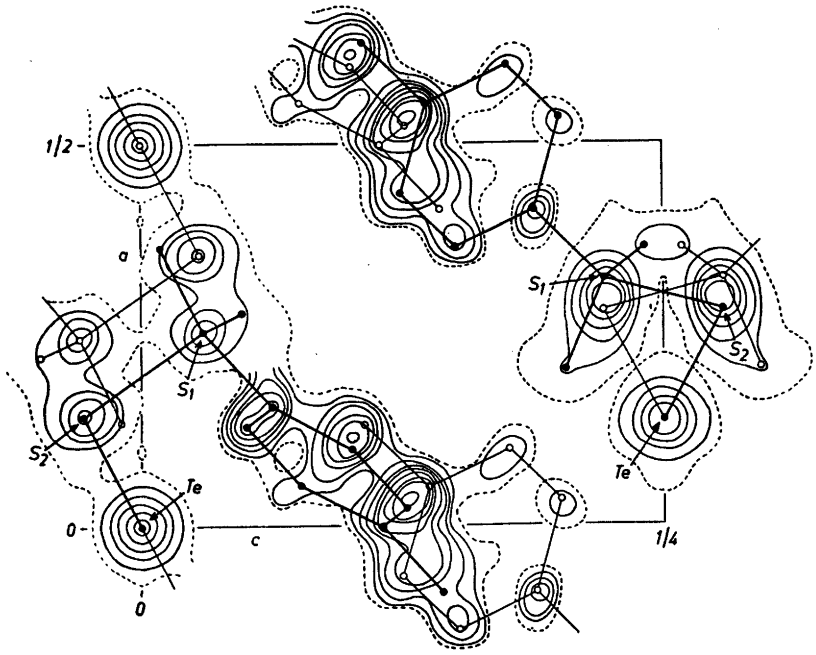

*Fig. 1. Electron density projection of tellurium di-*p*-toluenethiosulphonate along the* b *axis. The summations were made at 6° intervals along the* a *axis and 3° intervals along the* c *axis. The first contour line is at 6 e·Å^{-2} for the carbon atoms, 10 e·Å^{-2} for the sulphur and oxygen atoms and 15 e·Å^{-2} for the tellurium atom, with subsequent intervals of 1, 5, and 15 e·Å^{-2}, respectively. The 5-electron line is dashed.*

reflection, 204; this change brought about a marked improvement of the Fourier map with respect to oxygen and carbon atoms. The new map indicated some small adjustments, chiefly a slight rotation of the molecule as a whole about the twofold axis, and of the *p*-tolyl groups about the S—C bonds, so as to give the following atomic coordinates, in fractions of cell edges and referring to an origin at the intersection of a twofold rotation axis [010] and a twofold screw axis.

These coordinates, with a temperature factor of $B = 5.0$ Å^2, give a reliability factor of $R = 0.21$ for the *h0l* reflections.

The *h0l* Fourier map is shown in Fig. 1. The positions corresponding to the given coordinates are marked with dots (equivalent positions with rings) and lines are drawn to show the outline of the molecules. The bond lengths and angles of the S—S—Te—S—S chain are, Te—S_2 = 2.41 ± 0.04 Å, S_2—S_1 = 2.11 ± 0.04 Å,

	x	*y*	*z*
Te	0	0.136	0
S_2	0.145	0.283	—0.028
S_1	0.253	0.322	0.029
O_1	0.365	0.361	0.009
O_2	0.278	0.203	0.047
C_1	0.154	0.413	0.062
C_2	0.130	0.533	0.050
C_3	0.052	0.604	0.075
C_4	—0.003	0.555	0.113
C_5	0.021	0.435	0.126
C_6	0.099	0.363	0.100
C_7	—0.089	0.634	0.105

$\angle S_2TeS_2' = 96 \pm 3°$, $\angle TeS_2S_1 = 103 \pm 3°$, and the dihedral angle, $S_1S_2Te/S_2TeS_2' = 86°$. These data agree well with those found for tellurium dibenzenethiosulphonate.

The structure will not be refined further, but employed as a reference in the determination of the detailed structure of the

isomorphous crystals of triselenium di-*p*-toluenesulphinate. Work on the latter structure is in progress.

1. Öyum, P. and Foss, O. *Acta Chem. Scand.* **9** (1955) 1012.
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The Structure of Triclinic Barium Pentathionate Dihydrate

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The isolation of two crystalline modifications of barium pentathionate dihydrate, *viz.*, one orthorhombic, space group D_{2h}^{16}—*Pnma* with $Z = 4$, and one triclinic, was reported two years ago [1]. The structure of the orthorhombic crystals has later been published in detail [2], and a preliminary account of the structure of the triclinic dimorph is given below.

The unit cell dimensions of triclinic barium pentathionate dihydrate, $BaS(S_2O_3)_2 \cdot 2H_2O$, are: $a = 5.00$ Å, $b = 10.36$ Å, $c = 11.53$ Å, $\alpha = 109°$, $\beta = 98°$, $\gamma = 90°$. There are two molecules per unit cell, and the space group is C_i^1—$P\bar{1}$. The intensities of the $0kl$ and $h0l$ reflections were estimated visually from zero layer Weissenberg photographs taken with CuK radiation, and the structure was solved through Patterson and Fourier projections along the a and b axes, using in the initial stages the heavy atom technique. The $0kl$ Fourier map is shown in Fig. 1. The atomic coordinates, in fractions of corresponding cell edges and referring to the triclinic axes, are:

	x	y	z
Ba	0.749	0.211	—0.104
S_1	0.755	0.077	0.186
S_2	0.808	0.225	0.366
S_3	0.564	0.381	0.359
S_4	0.808	0.542	0.366
S_5	0.755	0.554	0.186
O_1	0.462	0.070	0.158
O_2	0.920	—0.031	0.210
O_3	0.874	0.140	0.108
O_4	0.462	0.551	0.158
O_5	0.920	0.676	0.210
O_6	0.874	0.432	0.108
$(H_2O)_1$	0.253	0.250	0.007
$(H_2O)_2$	0.603	0.131	—0.363

With a temperature factor of $B = 2.2$ Å^2, the reliability factor, $R = 0.18$ and 0.19, respectively, for the $0kl$ and $h0l$ reflections. The coordinates give the following dimensions of the sulphur chain, $S_1-S_2 = 2.12$ Å, $S_2-S_3 = 2.04$ Å, $S_3-S_4 = 2.04$ Å, $S_4-S_5 = 2.10$ Å (all $\pm$ 0.04 Å), $\angle S_1S_2S_3 = 107°$, $\angle S_2S_3S_4 = 107°$, $\angle S_3S_4S_5 = 106°$ (all $\pm$ 3°), and the dihedral angles, $S_1S_2S_3$/

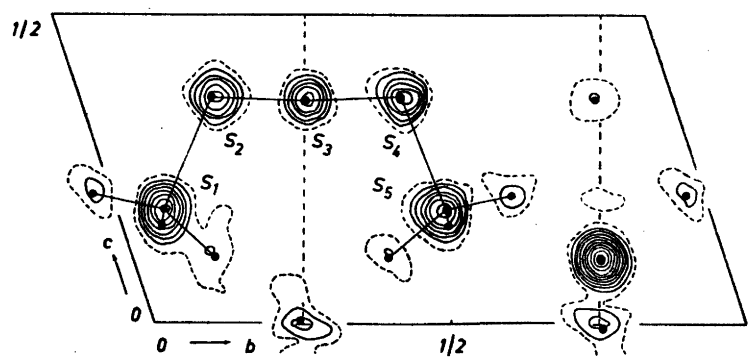

Fig. 1. Electron density projection of triclinic $BaS(S_2O_3)_2 \cdot 2H_2O$ *along the a axis. The atomic positions are marked with dots, and lines are drawn to show the outline of the pentathionate ion. The 5-electron line is dashed. Contour intervals: 10 e · Å^{-2} for the barium ion, and 4 e · Å^{-2} for sulphur and oxygen atoms and water molecules.*

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O_3	0.874	0.140	0.108
O_4	0.462	0.551	0.158
O_5	0.920	0.676	0.210
O_6	0.874	0.432	0.108
$(H_2O)_1$	0.253	0.250	0.007
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With a temperature factor of $B = 2.2$ Å^2, the reliability factor, $R = 0.18$ and 0.19, respectively, for the $0kl$ and $h0l$ reflections. The coordinates give the following dimensions of the sulphur chain, $S_1-S_2 = 2.12$ Å, $S_2-S_3 = 2.04$ Å, $S_3-S_4 = 2.04$ Å, $S_4-S_5 = 2.10$ Å (all $\pm$ 0.04 Å), $\angle S_1S_2S_3 = 107°$, $\angle S_2S_3S_4 = 107°$, $\angle S_3S_4S_5 = 106°$ (all $\pm$ 3°), and the dihedral angles, $S_1S_2S_3$/

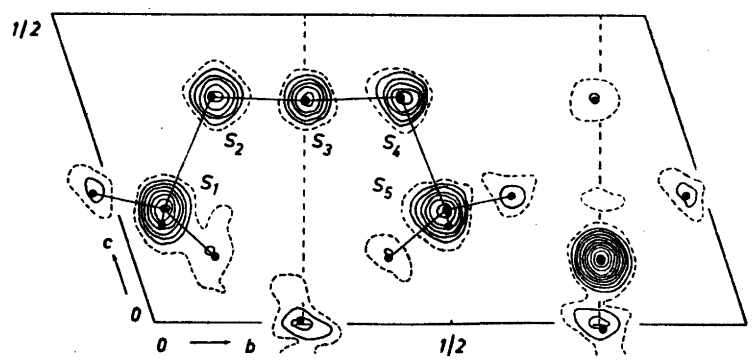

Fig. 1. Electron density projection of triclinic $BaS(S_2O_3)_2 \cdot 2H_2O$ *along the a axis. The atomic positions are marked with dots, and lines are drawn to show the outline of the pentathionate ion. The 5-electron line is dashed. Contour intervals: 10 e · Å^{-2} for the barium ion, and 4 e · Å^{-2} for sulphur and oxygen atoms and water molecules.*

$S_2S_3S_4 = 107°$ and $S_2S_3S_4/S_3S_4S_5 = 106°$. The slight differences between these values and those reported earlier [2] for the orthorhombic structure, are within the experimental errors.

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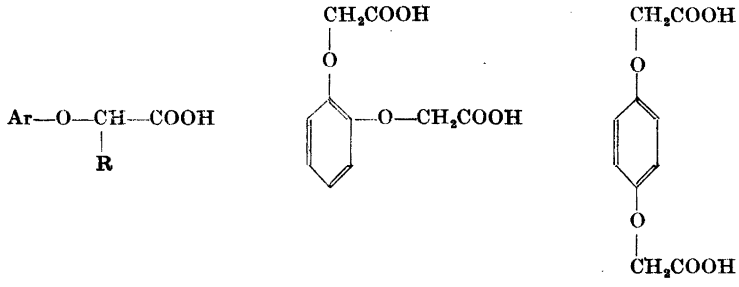
If the ether linkage in aryloxyalkylcarboxylic acids (I) is replaced by S, NH or CH_2 the plant growth-regulating activity is decreased [1,2]. This type of

linkage thus seems to be of great importance for the biological activity. However, the activity seems not to increase further if another —OCH(R)COOH side chain is introduced. The dioxyacetic acid derivatives of pyrocatechol (II) and hydroquinone (III) are inactive like phenoxyacetic acid [3].



I II III

The introduction of the second carboxylic group probably disturbs the balance between the hydrophilic and the lipophilic parts of the molecule. This H/L balance has been shown by Veldstra to be an important idea in dealing with structure-activity relations [4]. If an alkoxy group without carboxyl is introduced into phenoxyacetic acid the H/L balance is only slightly changed and the effects can be more unequivocally ascribed to the ether linkage. In fact Åberg has found that *m*- and *p*-methoxyphenoxyacetic acids are weak auxins, about 100 times less active than 2,4-D, while the *o*-derivative is an anti-auxin [3]. These results are contrary to the opinion that only halogens or alkyl groups are of importance in the study of structure-activity relations. It thus seemed important to investigate some alkoxy compounds and in this paper some halogenated guaiacoxyalkylcarboxylic acids will be described (Table 1).

A contributory cause for choosing guaiacol as starting material is that this phenol can be obtained by degradation of lignin. Except for vanillin, low molecular compounds are not prepared in a technical scale from this immense source of raw material. This is due not only to technical difficulties but also to difficulties in making use of the phenolic degradation products of lignin, *e. g.* guaiacol, creosol and pyrocatechol, which have been obtained from lignin in as high yield as 35 % [5]. For this reason and also in order to study structure-activity relations, further experiments are planned involving derivatives of vanillin, creosol, catechol and *iso*eugenol.

According to Beilstein, 2nd supplement, guaiacols should be numbered with the methoxy group in position 1 and the free hydroxy group in position 2. This system is inconvenient when applied to aryloxyalkylcarboxylic acids, which are usually numbered with the carboxylic acid chain in position 1. The latter system has been used here. Guaiacols named according to Beilstein's system have been placed within quotation marks.

The bromoguaiacols used as starting materials were obtained in good yields according to procedures already described in the literature [6,7]. 4,5-Dichloroguaiacol was prepared according to Peratoner and Ortoleva [8]. By a slight modification of their procedure the yield and purity of the product could be improved to an essential degree. "4-Chloroguaiacol" was prepared according to Jona and Pozzi [9]. These authors also prepared "5-chloroguaiacol" but their procedure was found to be very time-consuming and gave a very low yield. Peratoner has reported a liquid monochloroguaiacol of unknown structure obtained by the action of sulphurylchloride on guaiacol [8]. The chloroguaiacoxyacetic acid of this compound has now been prepared and was found to melt at 140°, only 3° below the guaiacoxyacetic acid derived from "4-chloroguaiacol". However, these two acids form an eutectic at 116° which proves that they are not identical. As the introduction of a second chlorine atom into guaiacol by means of sulphurylchloride leads to 4,5-dichloroguaiacol the product of Peratoner must contain mainly "5-chloroguaiacol". According to the literature it

Table 1. Halogenated guaiacoxyalkylcarboxylic acids.

Compound	Yield (%)		Recrystallized from	M. p. (°C)	Eq. wt.	
	crude	pure			calc.	found
Phenoxyacetic acids:						
2-Methoxy-4-chloro-	91	47	2 × 20% formic acid, 2 × toluene.	138 —140.0	216.6	217.1
» -5- »	66	43	20 % formic acid, toluene.	141.5—143.3	216.6	217.0
» -4,5-dichloro-	90	69	50 % formic acid, toluene.	156 —157.0	251.1	253.0
» -4-bromo-	97	51	40 % formic acid, toluene.	133 —134.8	261.1	262.0
» -5- »	89	70	50 % formic acid, toluene.	142 —143.8	261.1	261.1
α-Phenoxypropionic acids:						
2-Methoxy-4-chloro-	97	48	30 % formic acid, toluene.	103 —105.0	230.7	230.6
» -4,5-dichloro-	100	86	50 % formic acid, toluene-petr. ether.	123.5—124.0	265.1	265.4
» -4-bromo-	86	59	50 % formic acid, petroleum (85—110°)	111 —112.8	275.1	274.8
» -5- »	96	78	30 % formic acid, petroleum (85—110°)	102.5—103.5	275.1	275.4

should melt at 161° which however seems rather improbable as the 4-chloro isomer melts at 36°. It is possible that the melting point of the literature refers to the K-salt as was the case with "4-chloroguaiacol" when first reported [9,10]. In order to settle this question the direct chlorination of guaiacol will be further investigated.

Experimental. 4,5-Dichloroguaiacol. Sulphurylchloride (148.5 g, 1.1 mole) was slowly added (2 hours) to guaiacol (62 g, 1.0 mole). The mixture was allowed to stand for 3 hours when it solidified. The crude product was washed with petroleum ether and recrystallized from petroleum (b. p. 60—85°) yielding 81 g (84 %) 4,5-dichloroguaiacol, m. p. 62—70°. Further recrystallizations from the same solvent yielded 46 g (47 %), m. p. 71.5—73.0° (lit. 71—72°).

Guaiacoxyalkylcarboxylic acids. Sodium (1.15 g, 0.05 g-atom) was dissolved in abs. ethanol (40 ml). The appropriate phenol (0.05 mole) and ethyl chloroacetate (6.1 g, 0.05 mole) or ethyl α-bromopropionate (9.1 g, 0.05 mole) were added and the solution refluxed for 3 hours. Hydrolysis was performed by refluxing for 1 hour with 2 *N* NaOH (40 ml). The solution was acidified with 2 *N* HCl when the organic acid separated, in most cases as an oil which crystallized within 1 hour. The crude product was recrystallized as seen from Table 1.

Melting points. All melting points were determined with a hot stage microscope (Table 1). A contact preparation [11] of 2-methoxy-5-chlorophenoxyacetic acid and 2-methoxy-*eso*-chlorophenoxyacetic acid was studied with the same apparatus. The components are not isomorphic and the lowest melting point of the system, 116°, is thus an eutectic.

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The Inhibitory Effect of Diaminopurine Riboside on the Growth of *Ophiostoma*

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Institute of Physiological Botany, University of Uppsala, Uppsala, Sweden

The possible function of certain purines and purine ribosides as intermediates in the biosynthesis of nucleic acids has been a matter of discussion for several years. One way of gaining some insight into this problem is to study the response of purine-requiring mutants of microorganisms to various postulated intermediates. 2,6-Diaminopurine, free or in a ribosidic linkage, has been suggested as a possible precursor of nucleic-acid purines, and was therefore tested in experiments with various purine-less mutants as well as with wild type strains of the fungus *Ophiostoma multiannulatum*.

As was earlier shown [1], free diaminopurine is as capable as guanine in supporting the growth of the guanine-less mutants, and in contrast to what has been found in certain other organisms [2,3] it does not possess any inhibitory influence upon other *Ophiostoma* mutants or wild type strains.

Diaminopurine riboside, on the other hand, could not be utilized by any of the hypoxanthine-, adenine-, or guanine-less mutants, but proved to be a strong inhibitor of growth. In wild type strains a concentration of 30×10^{-6} *M* completely prevented growth, whereas 3×10^{-6} *M* still decreased the rate of growth considerably (Table 1). Diaminopurine riboside probably acts as an antimetabolite to adenosine, since this substance appeared more efficient than the other purine derivatives tested in counteracting the inhibition. A similar effect has been noted in recent studies on mammalian neoplastic tissues, where the toxicity of diaminopurine riboside can be blocked by adenosine (and adenine) [5,6].

As a general conclusion from the experiments with *Ophiostoma* it may be said that the results do not support the assumption of diaminopurine riboside being a normal intermediate in nucleic acid biosynthesis in this organism.

Table 1. Effect of 2,6-diaminopurine riboside (DAPR) on the growth of a wild type strain, No. 51, of Ophiostoma with and without a further addition of other purine derivatives. The fungus was cultivated in shake tubes (triplicates) as a conidial culture, and the growth was measured photometrically [4]. The figures in the table indicate the growth attained after two days in per cent of the control without any purines added.

DAPR 10^{-6} *M*	Further addition, each 50 μmoles/lit.					
	None	Diaminopurine	Adenine	Guanine	Adenosine	Guanosine
0	100	96	100	100	96	100
3	87	87	87	85	89	87
5	75	37	58	51	82	79
10	20	9.7	12	8.1	66	37
30	5.4	5.0	5.8	4.3	25	6.5
50	4.6	4.6	4.6	3.9	18	4.3

The author is greatly indebted to Dr. G. B. Brown, Sloan-Kettering Institute, New York, for the samples of diaminopurine and its riboside. The investigation was supported by a grant from *Eli Lilly and Co.*, Indianapolis, Ind.

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The Content of Polyglucose of Glycogenic Nature * in *Escherichia coli B* during Growth in Media Deficient in Nitrogen and Carbon

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During an investigation of *Escherichia coli B* cultivated by the continuous technique at different growth rates, it was

* For the sake of brevity called "glycogen".

Table 1.

Exp.	Time after inoculation in min	Number of cells/ml $\times 10^8$	"Glycogen" % of dry weight	Mg "glycogen" per liter of culture	"Glycogen"/ /cell mg $\times 10^{-12}$
A	0	0.75	2.1	0.3	3.8
	30	0.80	8.8	1.7	20.8
L.F.: NH_4Cl	90	1.5	8.4	3.3	32.8
C.S.: lactate	140	2.7	6.1	4.8	20.6
	180	4.5	9.3	10.4	37.6
	220	4.6	12.2	13.6	50.6
NH_4Cl	260	4.9	15.3	18.2	63.1
⟶ added	270	5.3	13.8	17.2	58.5
	325	8.5	7.5	14.2	33.4
	475	—	0.4	3.5	—
B	0	1.0	2.1	0.3	2.5
L.F.: lactate	90	0.74	7.8	1.5	20.3
C.S.: lactate	360	7.0	4.1	5.4	7.7
	420	10.0	2.7	5.7	5.7
	480	15.0	2.8	7.0	4.7
	600	16.0	4.6	12.0	7.5
lactate	660	16.0	5.7	15.5	9.7
⟶ added	720	16.0	6.4	17.2	10.7
	780	22.0	6.9	26.8	12.2
	840	26.0	5.4	25.2	9.7
C§ L.F.: NH_4Cl	0	1.2	6.6	1.3	10.4
C.S.: glucose	60	1.2	13.4	3.2	26.0
NH_4Cl	150	1.7	11.0	5.6	33.0
⟶ added	300	2.8	21.0	21.2	75.7
	375	5.9	11.9	24.6	41.6
D	0	1.1	1.9	0.3	2.5
L.F.: NH_4Cl	140	2.1	5.4	3.7	17.7
C.S.: lactate	300	6.3	18.2	17.5	27.8
No addition of L.F.	540	5.7 §§	21.4	25.2	44.9

L.F.: limiting factor. C.S.: carbon source.
§ After 200 min. the number of cells remained constant at 2.8×10^8 per ml.
§§ Difficult to count in the Buerker chamber because of the scanty contrast.

found that the nature of the limiting factor had a profound influence on the "glycogen" content of the cells. In order to obtain additional information, it was regarded as essential that also cells obtained in batchgrowth were investigated, where the growth was limited by the nitrogen source (NH_4Cl) or by the carbon source (sodium lactate).

Experimental. In two experiments (A and B) the culture volume was 30 litres. In the other two (C and D) it was 5 litres. The cultivation and harvesting procedures are previously described [1]. The main substrate was Friedleins synthetic medium containing Na-lactate as the carbon source and NH_4Cl as the nitrogen source [2]. In one experiment (C) glucose was the carbon source [3]. In the media deficient in nitrogen (A, C, and D), the NH_4Cl was 0.75×10^{-3} *M*. The carbon deficient medium (B) was 1.67×10^{-2} *M* with regard to lactic acid. After the initial lag and multiplication periods, the cells stopped to divide because of the deficiency of the medium. After an additional time of 1.5 hours in the nitrogen deficient cultures and of 4 hours in the carbon deficient one, an amount of the limiting factor was added to make the cultures (A, C, and B) 0.02 *M* and 0.1 *M*, respectively.

Table 2.

Exp.	Time after inoculation in minutes	Cold TCA-N mg per liter of culture	+ hot TCA-N per cell mg × 10^{-12}	Cold TCA-N / Hot TCA-N ratio
B	360	5.8	8.3	1.5
L.F.: Na-lactate	480	9.9	6.6	1.6
	540	11.3	6.6	1.6
	600	10.3	6.4	1.4
	660	10.0	6.3	1.3
	720	9.6	6.1	1.5
	721	sodium lactate added		
	780	13.1	5.9	0.7
	840	15.5	6.0	0.4

The "glycogen" was isolated and the glucose obtained from the "glycogen" as described in a paper to follow [4]. The trichloroacetic acid (TCA) extraction in experiment B was performed according to Schneider [5].

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The ratio between the nitrogen in the cold and the hot TCA extracts was higher than 1.3 during the whole carbon deficient period in experiment B, where Na-lactate was the limiting factor. After the addition of lactate the ratio rapidly decreased (Table 2).

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On the Correspondence between Thermocells and Isothermal Cells

HANS HOLTAN JR.* and JAN KROGH-MOE

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A theoretical relationship between the thermopotential difference of two pure thermocells and the corresponding isothermal cell has been derived recently by Holtan [1,2].

Consider for instance the thermocell

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For the differential thermopotential difference of this cell we write $(\Delta\varphi/\Delta T)_1$.

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For the differential potential difference of this cell we write $(\Delta\varphi/\Delta T)_2$.

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The Inhibitory Effect of Diaminopurine Riboside on the Growth of *Ophiostoma*

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The possible function of certain purines and purine ribosides as intermediates in the biosynthesis of nucleic acids has been a matter of discussion for several years. One way of gaining some insight into this problem is to study the response of purine-requiring mutants of microorganisms to various postulated intermediates. 2,6-Diaminopurine, free or in a ribosidic linkage, has been suggested as a possible precursor of nucleic-acid purines, and was therefore tested in experiments with various purine-less mutants as well as with wild type strains of the fungus *Ophiostoma multiannulatum*.

As was earlier shown [1], free diaminopurine is as capable as guanine in supporting the growth of the guanine-less mutants, and in contrast to what has been found in certain other organisms [2,3] it does not possess any inhibitory influence upon other *Ophiostoma* mutants or wild type strains.

Diaminopurine riboside, on the other hand, could not be utilized by any of the hypoxanthine-, adenine-, or guanine-less mutants, but proved to be a strong inhibitor of growth. In wild type strains a concentration of 30×10^{-6} *M* completely prevented growth, whereas 3×10^{-6} *M* still decreased the rate of growth considerably (Table 1). Diaminopurine riboside probably acts as an antimetabolite to adenosine, since this substance appeared more efficient than the other purine derivatives tested in counteracting the inhibition. A similar effect has been noted in recent studies on mammalian neoplastic tissues, where the toxicity of diaminopurine riboside can be blocked by adenosine (and adenine) [5,6].

As a general conclusion from the experiments with *Ophiostoma* it may be said that the results do not support the assumption of diaminopurine riboside being a normal intermediate in nucleic acid biosynthesis in this organism.

Table 1. Effect of 2,6-diaminopurine riboside (DAPR) on the growth of a wild type strain, No. 51, of Ophiostoma with and without a further addition of other purine derivatives. The fungus was cultivated in shake tubes (triplicates) as a conidial culture, and the growth was measured photometrically [4]. The figures in the table indicate the growth attained after two days in per cent of the control without any purines added.

DAPR 10^{-6} *M*	Further addition, each 50 μmoles/lit.					
	None	Diaminopurine	Adenine	Guanine	Adenosine	Guanosine
0	100	96	100	100	96	100
3	87	87	87	85	89	87
5	75	37	58	51	82	79
10	20	9.7	12	8.1	66	37
30	5.4	5.0	5.8	4.3	25	6.5
50	4.6	4.6	4.6	3.9	18	4.3

The author is greatly indebted to Dr. G. B. Brown, Sloan-Kettering Institute, New York, for the samples of diaminopurine and its riboside. The investigation was supported by a grant from *Eli Lilly and Co.*, Indianapolis, Ind.

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The Content of Polyglucose of Glycogenic Nature * in *Escherichia coli B* during Growth in Media Deficient in Nitrogen and Carbon

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During an investigation of *Escherichia coli B* cultivated by the continuous technique at different growth rates, it was

* For the sake of brevity called "glycogen".

Table 2.

Exp.	Time after inoculation in minutes	Cold TCA-N mg per liter of culture	+ hot TCA-N per cell mg × 10^{-12}	Cold TCA-N / Hot TCA-N ratio
B	360	5.8	8.3	1.5
L.F.: Na-lactate	480	9.9	6.6	1.6
	540	11.3	6.6	1.6
	600	10.3	6.4	1.4
	660	10.0	6.3	1.3
	720	9.6	6.1	1.5
	721	sodium lactate added		
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On the Correspondence between Thermocells and Isothermal Cells

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A theoretical relationship between the thermopotential difference of two pure thermocells and the corresponding isothermal cell has been derived recently by Holtan [1,2].

Consider for instance the thermocell

$$\underset{T}{Pt(H_2);\ HCl_{aq};}\ \underset{T+\Delta T}{(H_2)Pt} \qquad (1)$$

For the differential thermopotential difference of this cell we write $(\Delta\varphi/\Delta T)_1$.

Consider then the thermocell

$$\underset{T}{Ag;\ AgCl,\ HCl_{aq},}\ \underset{T+\Delta T}{AgCl;\ Ag} \qquad (2)$$

For the differential potential difference of this cell we write $(\Delta\varphi/\Delta T)_2$.

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According to the relation derived

$$(\Delta\varphi/\Delta T)_1-(\Delta\varphi/\Delta T)_2 = (\mathrm{d}\Delta\varphi/\mathrm{d}T)_{is} + (\Delta\varphi/\Delta T)_m \quad (3)$$

where $(\mathrm{d}\Delta\varphi/\mathrm{dT})_{is}$ is the temperature coefficient of the sothermal cell

$$Ag;\ AgCl,\ HCl_{aq};\ (H_2)Pt \quad (4)$$

We have hereby used the American sign convention. Note that the German sign convention was used in references 1 and 2. $(\Delta\varphi/\Delta T)_m$ is the differential thermopotential difference of the metallic thermocouple silver-platinum.

The system (1) has been investigated experimentally for m = 1.

Two vessels connected by a siphon was used. One vessel was submerged in a Dewar vessel maintained at 0° C by means of ice. The other was submerged in an ultrathermostat. The temperature of this vessel was registered by means of a mercury precision thermometer. The potential differences were measured by means of a potentiometer from Tinsley & Co, London, 1953. The electrodes were simple of the Hildebrand type. Freshly platinized platinum electrodes from Mattey & Co., Ltd, London, were used. The platinum wires were lead all way out to the potentiometer. The hydrogen gas was generated by electrolysis and bubbled through alkaline pyrogallol solution and then through 1 *M* hydrochloric acid.

It is well known that the hydrogen electrode of the Hildebrand type is unstable to some degree [3], however, different sets of electrodes were tried until a set was found that showed zero potential difference at zero temperature difference.

The differential potential difference of this cell was found to be +0.60 mV/degree at 25° C (positive sign because the cold electrode was found to be positive). Owing to the difficulties mentioned, the limits of error must be considered rather large, say ±0.03 mV/degree.

The differential thermopotential difference of the system (2) for m = 1 has been determined by Bernhardt and Crockford [4] to −0.08 mV/degree (mean value in the temperature interval 25−35° C), whereas Hall and Crockford [5] found 0.02 mV/degree in the temperature interval 0−25° C.

We see that the thermopotential difference according to the figures listed above has an appreciable temperature dependence (especially for systems containing hydrochloric acid). As we want to consider the value at 25° C, the value −0.08 is somewhat high (probably of the order 0.01−0.02 mV/degree). Combining, however, this value with the experimental value for the thermocell (1), we get

$$(\Delta\varphi/\Delta T)_1 - (\Delta\varphi/\Delta T)_2 = 0.68 \text{ mV/degree.}$$

For the temperature coefficient of the isothermal cell (4) we calculate after data given by Eastman and Milner [5], Eucken, Clusius and Woitinek [7], Uhlich [8] and Rossini *et al.*[9] for m = 1 at 25° C

$$(\mathrm{d}\Delta\varphi/\mathrm{d}T)_{is} = 0.66 \text{ mV/degree}$$

As the thermoelectric power of the thermocouple silver-platinum is small (+0.01 mV/degree [10]), we see that relation (3) holds within the limits of experimental error.

We may get another test of equation (3) by comparing the two thermocells

$$\underset{T}{Hg;Hg_2Cl_2},\ \underset{T+\Delta T}{MCl_{aq};\ Hg} \quad (5)$$

and the cell (2).

The thermopotential difference of system (5) was determined experimentally for HCl, m = 1, to 0.30 mV/degree at 25° C.

We then obtain

$$(\Delta\varphi/\Delta T)_1 - (\Delta\varphi/\Delta T)_5 = 0.30 \text{ mV/degree}$$

which favorably compares with 0.32 mV/degree calculated for the corresponding isothermal cell. In addition to data already referred to, we have used data listed by Kelly [11,12].

The system (5) may also be compared with system (2). Richards [13] investigated the cell (5) in the temperature interval 0−30° C. These results may then be compared with those of Crockford and Hall [5] for the temperature interval 0−25° C without introducing any large error.

In Table 1 we have listed the difference between the thermopotential differences of the systems (5) and (2).

Table 1.

Chloride		$(\Delta\varphi/\Delta T)_5 - (\Delta\varphi/\Delta T)_1$
HCl NaCl KCl	m = 1	0.31 0.32 Average: 0.32 0.32 mV/degree
HCl NaCl KCl	m = 0.01	0.33 0.32 Average: 0.30 0.32 mV/degree

For the isothermal cell

$$Ag;\ AgCl,\ MCl_{aq},\ Hg_2Cl_2;\ Hg \qquad (6)$$

we obtain $(d\Delta\varphi/dT)_{is} = 0.34$ mV/degree at 25°C.

As the thermoelectric force of the thermocouple silver-mercury is small (about 0.01 mV/degree [10]), we again find that the relation (3) holds within the limits of experimental error.

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Oxidative Stress Relaxation of Natural Rubber Vulcanized with Di-*Tertiary*-Butyl Peroxide

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It has been shown by Farmer and Moore [1] that natural rubber can be vulcanized with di-*tert.*-butyl peroxide (DTBP). Presumably the free radicals formed by the unimolecular decomposition of the peroxide abstract some of the more labile (*e. g.* α-methylenic) hydrogen atoms leading to direct C—C crosslinks between the rubber molecules, *tert.*-butanol and acetone being the main reaction products.

This preliminary communication presents some of the results of an investigation of the oxidative stress relaxation of the following types of DTBP vulcanizates: (A). First grade pale crepe, DTBP, and some carbon black (MPC) mixed on the mill and vulcanized in a press. The carbon black was added to minimize the deleterious effect of impurities. (B). Purified rubber vulcanized: 1. In aqueous heating media. 2. In the press. 3. In DTBP vapour.

(A). In Fig. 1 is plotted on a logarithmic scale the relaxation curves for a vulcanizate with 20 % MPC vulcanized for 15 minutes at 160° and relaxed in air at 90—140° (modulus 2.57 kg/cm² at an extension ratio of 1.2). A rapid initial rate of relaxation due primarily to peroxidic reaction products, was eliminated by preheating the samples *in vacuo* for 30

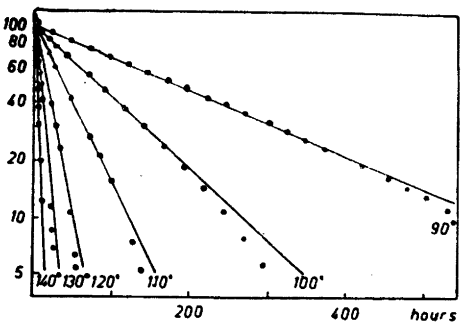

Fig. 1. Relaxation curves. Ordinate: % of original stress. Abscissa: hours.

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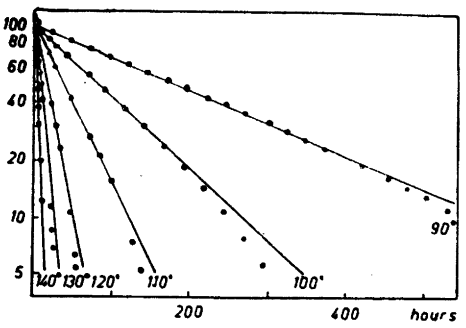

Fig. 1. Relaxation curves. Ordinate: % of original stress. Abscissa: hours.

min. at 150°. This treatment also serves to remove the physical relaxation. From the relaxation curves shown in Fig. 1 an energy of activation of 22.7 kcal is calculated for the relaxation process. The same relaxation rates are obtained when only 5 % MPC is used. The relaxation rate is nearly inversely proportional to the initial modulus of the sample, corresponding to a rate of oxidation nearly independent of the degree of vulcanization. With no preheating *in vacuo* there would also in addition to the normal initiation of oxidation chains by hydroperoxide decomposition, be an initiation by some non-volatile peroxidic materials formed during the vulcanization process and the amount of which will depend on the degree of vulcanization.

When approximately 75 % of the stress is relaxed, the relaxation curves of the preheated samples start deviating from linearity, apparently mainly because of the consumption of natural antioxidant in the oxidation. A closer examination shows that this occurs somewhat earlier when the degree of vulcanization is higher, corresponding to a consumption of natural antioxidant during vulcanization with DTBP. Acetone extraction of the samples before heat-treatment *in vacuo* has practically no influence on the relaxation rates. The antioxidants left are therefore probably proteins and possibly some of the normally extractable antioxidants which have been chemically bound to the rubber during the vulcanization.

The rate of crosslinking was measured by intermittently subjecting the relaxing sample to a small additional stress and measuring the corresponding increase in elongation. This method gives (as confirmed by tests on a pure gum sulphur vulcanizate) the same value for the rate of crosslinking as do the ordinary "intermittent stress relaxation" measurements on a separate sample, and the former method seem to have certain advantages. The rate of crosslinking was found to be 25—30 % of the rate of scission in the whole temperature range studied, *i. e.* with the same energy of activation. If the samples were acetone extracted, however, the rate of crosslinking was 45—50 % at 90° and reached 25—30 % at the higher temperatures. This may be a combined effect of the acetone-extractable substances acting as a plasticizer and the crosslinking capacity of the carbon black, although there was only a small difference between the effect when 20 % and when 5 % MPC was used.

(B). In order to avoid the use of additives such as carbon black a purified rubber was used in the following experiments, in which the effect of the degree and mode of vulcanization and of the pretreatment of the samples on their oxidation and relaxation behaviour was studied. The rubber was extracted from first grade pale crepe by a modification of the method of Bloomfield and Farmer [2] and should according to this be free of the "oxide fraction". The nitrogen content was about 0.035 %. Films of 0.05 mm thickness were prepared from solution and vulcanized at 150° for 30 min.

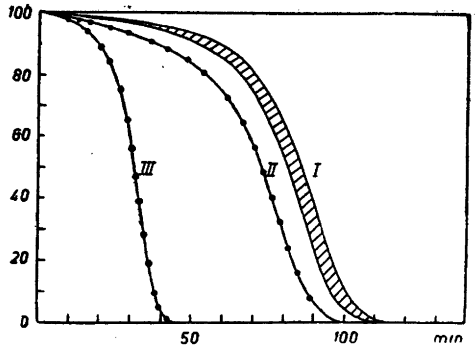

Fig. 2. Relaxation curves. Ordinate: % of original stress. Abscissa: minutes.

By the vulcanization in distilled water in an oxygen free atmosphere followed by extraction with acetone, the peroxidic material formed in the samples was still sufficient to be the dominating oxidation chain initiator at the beginning of the relaxation process. This peroxidic material seems to be chemically combined with the rubber and can preferably be destroyed by heating the sample *in vacuo*. Results of experiments on samples in which such a more nearly complete removal of the

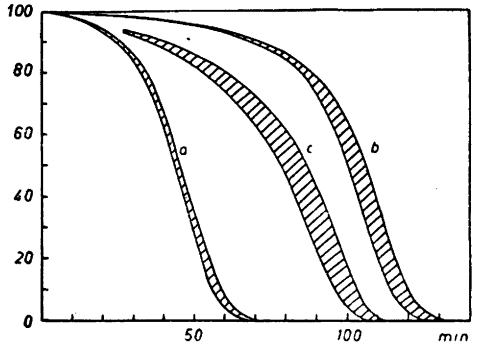

Fig. 3. Relaxation curves. Ordinate: % of original stress. Abscissa: minutes.

peroxidic material has been carried out, will be published later. In the experiments described here a relatively mild heat treatment of 10 min. at 120° was used. This reduced the initial relaxation rate by approximately 50 %. The hatched area I shown in Fig. 2 covers the relaxation curves (on a linear scale) of eight vulcanizates of modulus varying from 2.4 to 4.7 kg/cm² (at an extension ratio of 1.25), the upper curve corresponding to the higher modulus. Relaxation was carried out in dry oxygen at 90°. Without preheating, a modulus of 2.7 gives the relaxation curve II. Several factors play a part in determining the dependence of the curves I on modulus: The dependence of the concentration of the peroxidic material on the degree and conditions of vulcanization, the dependence of the oxidation rate on this concentration, and the consumption of natural antioxidant as a function of degree and mode of vulcanization.

The curve III represents a preheated sample of modulus 2.7 kg/cm² relaxed at 100°. The activation energy calculated from the relaxation curves differs little from the value obtained for the inhibited relaxation of the whole crepe vulcanizates described above. In accordance with the network theory the amount of oxygen absorbed (preliminary gravimetric measurements) at 99 % relaxation is proportional to the degree of vulcanization. There was no measurable crosslinking at 90°.

Fig. 3 shows the relaxation (at 90°) of samples vulcanized a) in water and with 5 % stearic acid in the rubber, b) in 2 *N* NaOH, and c) in pure DTBP vapour. (The ranges of moduli covered by the areas shown are not as large as in Fig. 2.) Apparently the stearic acid promotes the deactivation of the proteins, giving a less inhibited reaction, whereas the addition of NaOH to the water mainly serves to reduce the water concentration in the rubber, thus reducing the rate of deactivation by the DTBP. So efficient is this osmotic effect that a dry sample vulcanized in a press gave exactly the same relaxation curve.

It is interesting to observe the effect of incorporating 0.2 % copper stearate in the samples of a) before preheating. There is a marked reduction in the initial relaxation rate, presumably because of a more complete destruction of the peroxidic material *. In the later stages, however, the catalytic and antioxidant-inactivating effect of the copper stearate becomes apparent, giving a much faster rate than uncatalyzed samples.

By the vulcanization in DTBP vapour for 30 min. at 150°, the rubber became seriously overvulcanized at the surface. As should be expected, the curves c) in Fig. 3 show that the antioxidant-free stage is reached comparatively late in this case.

This investigation is being completed and extended to comprise accurate oxygen absorption measurements in order that the relation between this pure scission reaction and the detailed shape of the relaxation curve may be studied.

This work has been made possible by the kind assistance and financial support from *The British Council*, *The Rubber Development Board*, *The Rubber Producers Research Association*, *The Norwegian Council for Scientific and Industrial Research* and the *"Fridtjof Nansens Fond"*.

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* *Note added in proof:* This marked effect of copper stearate indicates that these peroxidic materials are rubber hydroperoxides. They may possibly be formed at the moment the cooled sample (with a certain amount of free radicals) is removed from the press or the autoclave and exposed to the air.

Separation of Corticosteroids by Countercurrent Distribution

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The separation of adrenal cortical steroids by physical methods has been attempted mainly by paper chromatography [1,2]. Countercurrent distribution has been applied by Voigt [3] in order to fractionate some nitrogen-containing, highly polar substances in adrenocortical extracts, supposed to be of steroidal nature. Talbot *et al.*[4] have recently reported the use of countercurrent distribution for fractionation of urinary corticosteroids. They seem, however, not to have achieved a satisfactory separation. Engel *et al.*[5] found that a 150 transfer countercurrent distribution was insufficient to separate urinary metabolites of corticosterone. The present experiments show that most corticosteroids may be

peroxidic material has been carried out, will be published later. In the experiments described here a relatively mild heat treatment of 10 min. at 120° was used. This reduced the initial relaxation rate by approximately 50 %. The hatched area I shown in Fig. 2 covers the relaxation curves (on a linear scale) of eight vulcanizates of modulus varying from 2.4 to 4.7 kg/cm² (at an extension ratio of 1.25), the upper curve corresponding to the higher modulus. Relaxation was carried out in dry oxygen at 90°. Without preheating, a modulus of 2.7 gives the relaxation curve II. Several factors play a part in determining the dependence of the curves I on modulus: The dependence of the concentration of the peroxidic material on the degree and conditions of vulcanization, the dependence of the oxidation rate on this concentration, and the consumption of natural antioxidant as a function of degree and mode of vulcanization.

The curve III represents a preheated sample of modulus 2.7 kg/cm² relaxed at 100°. The activation energy calculated from the relaxation curves differs little from the value obtained for the inhibited relaxation of the whole crepe vulcanizates described above. In accordance with the network theory the amount of oxygen absorbed (preliminary gravimetric measurements) at 99 % relaxation is proportional to the degree of vulcanization. There was no measurable crosslinking at 90°.

Fig. 3 shows the relaxation (at 90°) of samples vulcanized a) in water and with 5 % stearic acid in the rubber, b) in 2 *N* NaOH, and c) in pure DTBP vapour. (The ranges of moduli covered by the areas shown are not as large as in Fig. 2.) Apparently the stearic acid promotes the deactivation of the proteins, giving a less inhibited reaction, whereas the addition of NaOH to the water mainly serves to reduce the water concentration in the rubber, thus reducing the rate of deactivation by the DTBP. So efficient is this osmotic effect that a dry sample vulcanized in a press gave exactly the same relaxation curve.

It is interesting to observe the effect of incorporating 0.2 % copper stearate in the samples of a) before preheating. There is a marked reduction in the initial relaxation rate, presumably because of a more complete destruction of the peroxidic material *. In the later stages, however, the catalytic and antioxidant-inactivating effect of the copper stearate becomes apparent, giving a much faster rate than uncatalyzed samples.

By the vulcanization in DTBP vapour for 30 min. at 150°, the rubber became seriously overvulcanized at the surface. As should be expected, the curves c) in Fig. 3 show that the antioxidant-free stage is reached comparatively late in this case.

This investigation is being completed and extended to comprise accurate oxygen absorption measurements in order that the relation between this pure scission reaction and the detailed shape of the relaxation curve may be studied.

This work has been made possible by the kind assistance and financial support from *The British Council*, *The Rubber Development Board*, *The Rubber Producers Research Association*, *The Norwegian Council for Scientific and Industrial Research* and the *"Fridtjof Nansens Fond"*.

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Table 1. Partition coefficients of corticosteroids in different solvent systems evaluated from countercurrent distribution data.

A: Benzene/Water (22.0° C)
B: Benzene/Water (27.0° C)
C: Benzene/50 % Methanol + 50 % Water (22.0° C)
D: Benzene/50 % Ethanol + 50 % Water (22.0° C)
E: n-Hexane/Water (22.0° C)
F: 20 % Ethanol + 80 % Water; 0.05 % (w/v) NaCl/50 % Chloroform + 50 % n-Hexane (21.0° C)
G: 60 % n-Hexane + 40 % Benzene/5 % Ethanol + 95 % Water (22.0° C)
H: 20 % Ethanol + 80 % Water; 0.05 % (w/v) NaCl/70 % Carbon tetrachloride + 30 % n-Hexane (22.0° C)
I: 20 % Ethanol + 80 % Water; 0.05 % (w/v) NaCl/Carbon tetrachloride (23.0° C)

Corticosteroid	Solvent system *								
	A	B	C	D	E	F	G	H	I
Δ^4-Pregnene-11β, 17α, 21-triol-3,20-dione	0.32	0.39	0.14	0.30		2.57			
Δ^4-Pregnene-17α, 21-diol-3,11, 20-trione	0.92	1.01	0.26	0.64		0.54			
Δ^4-Pregnene-11β, 21-diol-3,20-dione	10.1		1.57	1.27			1.08	4.26	1.86
Δ^4-Pregnene-17α, 21-diol-3,20-dione	9.0	10.2	1.22	1.63			1.13	1.94	0.79
Δ^4-Pregnene-21-ol-3,11,20-trione			2.6	2.23	0.03		6.1		
Δ^4-Pregnene-21-ol-3,20-dione			16	7	2.45				

* The composition of the biphasic systems is described by the volumes of the pure components used for each phase.

separated in suitable biphasic systems by 24 to 48 transfers. Only the six conventional corticosteroids are included in this investigation. Aldosterone was not available.

The partition coefficients (K) for different systems of solvent pairs were calculated from the countercurrent distribution data according to the procedure of Way and Bennett [6] (Table 1). A fairly good separation is obtained if the separation factor, β, *i. e.* the ratio of higher to lower K of a pair of solutes, exceeds 3 in a 24 transfer countercurrent distribution and if it exceeds 2.6 in a 48 transfer distribution. No single system is efficient enough in separating all six corticosteroids investigated. The separation of Δ^4-pregnene-11β, 21-diol-3,20-dione and Δ^4-pregnene-17α, 21-diol-3,20-dione did not fulfil the abovementioned demands. The most favourable β value was obtained in system H (Table 1), $\beta = 2.35$. However, a reasonably simple differentiation is obtained in this case by applying the Porter-Silber reaction [7,8] — in which Δ^4-pregnene-17α, 21-diol-3,20-dione reacts and Δ^4-pregnene-11β, 21-diol-3,20-dione does not.

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Enzymic Synthesis of Xanthosine- and Guanosine-5-phosphate from Inosine-5-phosphate

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The studies of Greenberg and Buchanan *et al.*[1,2] have established IMP-5 * as a key intermediate in the biosynthesis of hypoxanthine in pigeon liver and considerable information has been obtained regarding the synthesis of this substance from smaller molecules *via* the formation of AIC-ribotide. Until recently very little was known, however, about the possible role of IMP-5 in the biosynthesis of other purine nucleotides. Carter and Cohen [3,4] have recently obtained evidence for a transformation of AIC-ribotide (or IMP-5) to AMP-5 by way of succinyl-AMP-5. With the discovery of this interesting reaction a connection has been established between IMP-5 and the biosynthesis of adenine nucleotides.

In view of the possibility that IMP-5 might be an intermediate in the biosynthesis of other purine nucleotides a study of the metabolism of this substance in pigeon liver extracts was carried out. When IMP-5-^{14}C was incubated with a dialyzed extract of acetone-dried pigeon liver in the presence of DPN, ATP, PGA or HDP, L-glutamine and L-glutamate, Mg^{++}-ions and phosphate buffer pH 7.4, two new radioactive peaks were obtained after chromatography on Dowex-2 with formic acid according to the method of Hurlbert *et al*[5]. These two peaks were tentatively identified as guanosine-5-phosphate and xanthosine-5-phosphate, respectively. Their identification rests on the following points: 1) A positive analysis for ribose and phosphate; 2) Acid hydrolysis yields guanine and xanthine, respectively, that can be crystallized to constant radioactivity after addition of authentic guanine or xanthine; 3) Complete dephosphorylation with *Crotalus* venom 5-phosphatase.

Further studies showed that the synthesis of GMP-5 occurred in two steps. In the first one, IMP-5 was oxidized to XMP-5 in the presence of DPN and phosphate buffer. ATP did not stimulate the reaction. If DPN was omitted there was no formation of XMP-5 (Table 1).

Table 1.

Expt. No.	Modifications of the medium		μmoles of XMP-5 formed
	DPN Nicotinamide	ATP HDP	
14	+	+	0.19
15	—	+	0.00
16	+	—	0.33
20			0.44

In expts. 14—16 a dialyzed extract of 500 mg acetone-dried pigeon liver was incubated at 37° C for one hour with 1 μmole IMP-5-^{14}C, 4 μmoles ATP, 100 μmoles HDP, 20 μmoles DPN, 120 μmoles nicotinamide, 150 μg pyridoxal phosphate, 120 μmoles L-aspartate, 240 μmoles L-glutamate, 60 μmoles L-glutamine, 120 μmoles NH_4Cl, 120 μmoles $MgSO_4$ and 1 000 μmoles K-phosphate buffer pH 7.4 with the modifications indicated in the table. Final volume 20 ml.

In expt. 20 a dialyzed extract was incubated under the same conditions with 1 μmole IMP-5-^{14}C, 20 μmoles DPN, 120 μmoles nicotinamide and 1 000 μmoles K-phosphate buffer pH 7.4. Final volume 15 ml.

When XMP-5-^{14}C obtained from this reaction was incubated with the extract in the presence of ATP, PGA, L-glutamine and L-glutamate, Mg^{++}-ions and phosphate buffer GMP-5 was obtained. Under these conditions no formation of IMP-5 could be detected. Further experiments indicated that with L-glutamine as amino group donor optimal synthesis of GMP-5 was obtained at lower concentrations of the amino acid than in corresponding experiments with L-glutamate. (Fig. 1) It was therefore tentatively concluded that

* The following abbreviations are employed: AIC-ribotide = 4-amino-5-imidazole-carboxamide ribotide, AMP-5 = adenosine-5-phosphate, ATP = adenosine triphosphate, DPN = diphosphopyridine nucleotide, GMP-5 = guanosine-5-phosphate, HDP = hexose diphosphate, IMP-5 = inosine-5-phosphate, PGA = 3-phosphoglyceric acid, XMP-5 = xanthosine-5-phosphate.

L-glutamine rather than L-glutamate was the specific amino group donor. Substituting L-aspartate or NH_4Cl for L-glutamine gave a very poor yield of GMP-5. When ATP was omitted no synthesis of GMP-5 was obtained (Table 2).

Table 2.

Expt. No.	Modifications of the medium					μmoles of GMP-5 formed
	ATP PGA	L-aspartate	L-glutamate	L-glutamine	NH_4Cl	
23	+	+	+	+	+	0.17
24	—	+	+	+	+	0.00
29	+	+	—	—	—	0.01
30	+	—	+	—	—	0.09
31	+	—	—	+	—	0.14
32	+	—	—	—	+	0.02

In expts. 23—24 a dialyzed extract of 500 mg acetone-dried pigeon liver was incubated at 37° C for one hour with 0.36 μmoles XMP-5-^{14}C, 2 μmoles ATP, 100 μmoles PGA, 200 μg pyridoxal phosphate, 120 μmoles L-aspartate, 240 μmoles L-glutamate, 60 μmoles L-glutamine, 120 μmoles NH_4Cl, 120 μmoles $MgSO_4$ and 1 000 μmoles K-phosphate buffer pH 7.4 with the modifications indicated in the table. Final volume 20 ml.

In expts. 29—32 a dialyzed extract was incubated under the same conditions with 0.20 μmoles XMP-5-^{14}C, 2 μmoles ATP, 100 μmoles PGA, 200 μg pyridoxal phosphate, 120 μmoles $MgSO_4$, 1 000 μmoles K-phosphate buffer pH 7.4 and 100 μmoles of either L-aspartate, L-glutamate, L-glutamine or NH_4Cl. Final volume 15 ml.

In view of these findings it seems justified to assume that the biosynthesis of GMP-5 from IMP-5 in pigeon liver goes by way of an oxidation to XMP-5 and a subsequent amination of this substance (eqn. 1).

$$\text{IMP-5} \xrightarrow{\text{DPN}} \text{XMP-5} \xrightarrow[\text{ATP}]{\text{L-glutamine}} \text{GMP-5} \quad (1)$$

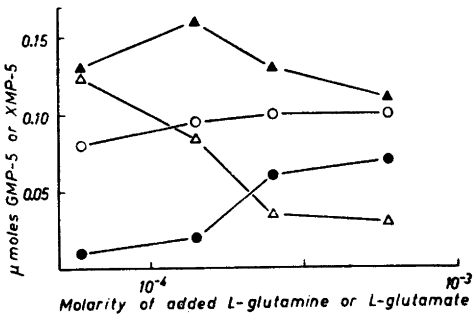

Fig. 1. Dependence of GMP-5 synthesis on L-glutamine or L-glutamate.

—○—○— *GMP-5 synthesized in experiments with L-glutamine.*
—△—△— *XMP-5 reisolated in experiments with L-glutamine.*
—●—●— *GMP-5 synthesized in experiments with L-glutamate.*
—▲—▲— *XMP-5 reisolated in experiments with L-glutamate.*

A dialyzed extract of 300 mg acetone-dried pigeon liver was incubated at 37° C for one hour with 0.30 μmoles XMP-5-^{14}C, 2 μmoles ATP, 50 μmoles PGA, 200 μg pyridoxal phosphate, 120 μmoles $MgSO_4$, 1 000 μmoles K-phosphate buffer pH 7.4 and L-glutamine or L-glutamate as indicated in the figure. Final volume 13 ml.

A full report of these findings will be given later.

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Paperelectrophoretic Studies on Material from Encapsulated *Bacillus anthracis*

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The earlier presumption of a complex composition of the capsule from *Bacillus anthracis* by Nordberg and Thorsell [1] seems to be confirmed in these paperelectrophoretic studies combined with different dyeing methods.

Materials and methods. *In vivo* encapsulated *B. anthracis* microbes were isolated from rabbits according to the method of Smith *et al.*[2] About 0.5 ml of the microbes were suspended in 25 ml of dist. water and left at 20° C until the somata were freed from their capsules. This procedure was followed by microscopic examination of smears stained with Löffler's methylene blue according to Heim [3]. The cell bodies retained their normal staining properties. The suspension obtained was centrifuged and the supernatant was evaporated in vacuum at 30° C to a volume of about 0.5 ml to form the "capsular material".

Hemolyzed rabbit blood was prepared by diluting normal rabbit blood with one volume of dist. water. The substances isolated from *B. anthracis in vivo* (poly-D-glutamic acid and polysaccharide) by Smith and Zwartou [4] were used as 1 % w/v in dist. water.

These materials were used in equivalent amounts for paper electrophoretic studies at pH 5.3 (0.1 *M* acetate buffer-solution), pH 6.5 (0.1 *M* phosphate buffer-solution) and pH 8.6 (veronal-acetate buffer-solution according to Michaelis).

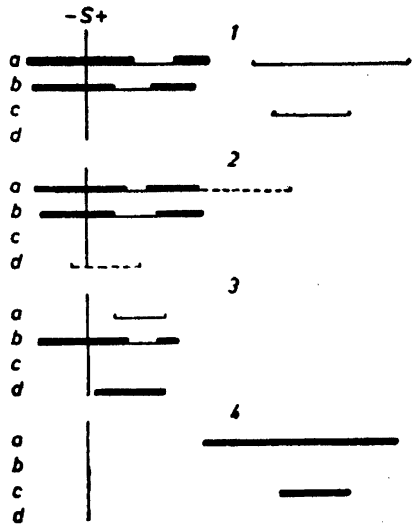
After drying at 105° C for half an hour the papers were dyed with

1) chlorine dioxide-benzidine according to Reindal and Hoppe [5].

2) Amidoblack 10 B according to Grassmann and Hannig [6] with the modification described in "Type LKB 3276 Paper Electrophoresis Equipment. Instructions for use" (AB. LKB, Sweden).

3) periodic acid-fuchsin sulphurous acid according to Wunderly and Piller [7].

4) Löffler's methylene blue, using a modification of the method described by Strange and Harkness [8], whereby the paperstrips were washed twice in 80 % ethanol, treated with 0.02 *M* hydrochloric acid in 90 % ethanol, washed in ethanol, dried and placed in methylene blue solution for 20 minutes.



Symbols: 1 = Chlorine-dioxide-benzidine. 2 = Amidoblack 10 B. 3 = Periodic acid-fuchsin-sulphurous acid. 4 = Löffler's methylene blue. a = B. anthracis "capsular material". b = Hemolyzed rabbit blood. c = B. anthracis poly-D-glutamic acid. d = B. anthracis polysaccharide. S = Starting line.

Results at pH 5.3 are shown in Fig. 1. The broader lines indicate a relatively greater staining intensity.

Discussion. It appears from Fig. 1 that *B. anthracis* "capsular material" contains an anodically rapidly migrating fraction stainable by the chlorine dioxide-benzidine method in the same way as the poly-D-glutamic acid isolated from *B. anthracis* (1 a, 1 c). Other *B. anthracis* components stainable by chlorine dioxide-benzidine or amidoblack in the "capsular material", perhaps, for example, the very weakly staining fraction seen in 2 a, are difficult to recognize as they, due to interferences, may be concealed by material from hemolyzed blood (1 a, 1 b; 2 a, 2 b).

The attempt to demonstrate other capsular components in the "capsular material" through the use of periodic acid — fuchsin sulphurous acid and methylene blue stain-

ing methods show that the only fraction of the "capsular material" stainable with methylene blue is the highly negatively charged one (4 a). This seems to indicate the occurrence of *B. anthracis* poly-D-glutamic acid (4 c). The more widespread area of the "capsular material" fraction could be due to various interferences (*cf.* 1 a, 2 a), at least one of which Cromartie *et al.*[9] observed in their electrophoretic investigations on material from anthrax oedema and which is perhaps visualized in the very weakly staining component of the "capsular material" (2 a). The slowly migrating fractions of the "capsular material" show a fuchsin-stainable component (3 a) almost at identical position with isolated *B. anthracis* polysaccharide (3 d). As the "capsular material" used seems to contain real capsular material and very little or no somatic material and as the other fuchsin-stainable components from hemolyzed blood (3 b) are not visible (3 a) this could mean that the carbohydrate component proposed for the *B. anthracis* capsule is identical with or similar to the isolated *B. anthracis* polysaccharide.

We want to express our sincere gratitude to Prof. H. Hedström, Prof. K. Sjöberg and Doc. H. Svensson, Stockholm, Sweden, for their interest and valuable help, and to Dr. H. Smith, Porton, England, for his kind gift of *B. anthracis* poly-D-glutamic acid and polysaccharide.

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Glucosamine and Galactosamine in Human Serum Fractions

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The physiology of the protein-bound carbohydrates is little known and even the knowledge about the chemical composition of the carbohydrate complex is lacking. In this work an investigation on the aminosugars is made.

Determination of the ratio galactosamine: glucosamine was carried out with the method given by Gardell [1] and hexosamines were determined by the Elson and Morgan method as worked out by Blix [2]. The electrophoretic separation of proteins was carried out by means of paper electrophoresis followed by elution of the fractions. The mucoproteins were isolated according to Winzler *et al.*[3]. The mucoprotein preparation can be electrophoretically divided into three mucoproteins, named MP-1, MP-2 and MP-3 (Mehl *et al.*[4]) MP-1 has the same mobility at pH 8.6 as α_1-globulin, the other two run slower.

In Table 1 the results of the galactosamine: glucosamine determinations are given as well as the hexosamine content in per cent of the protein.

It is seen that the ratio is about 1 : 18 for normal sera as well as for γ-globulin and MP-1. MP-2 and MP-3 have not yet been isolated in sufficient quantities for analysis but on the base of the values for MP-1 and for MP-1+2+3 there is indirect evidence for a high galactosamine content.

The α_2- and β-globulins can each be divided into two fractions, one precipitable with trichloroacetic acid (9 %) or with perchloric acid (0.6 *M*) (fraction A). In the filtrates remains a fraction precipitable with phosphotungstic acid (fraction B). The amount of fraction B is about 10—20 % of the whole α_1- or β-globulins. The galactosamine:glucosamine ratio of fraction A was for α_1-globulin 1:17.4 and for β-globulin 1:17.8. Thus it is seen that galactosamine and glucosamine are present in most fractions in a constant ratio of 1:18 but the mucoproteins following α_1- and β-globulin have a higher content of galactosamine.

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Table 1. The content of hexosamine in serum protein fractions and the relative amounts of galactosamine and glucosamine.

	Hexosamine, % of protein	Galactosamine μg	Glucosamine μg	Gal./Gluc. ratio
Normal serum 1		19.6	366.6	1 : 18.7
Normal serum 2		29.9	533.1	1 : 17.8
MP-1	11.5	18.9	335.8	1 : 17.8
MP-1+2+3	8.9	47.5	417.5	1 : 8.8
α_2-glob.	3.76	48.2	751.2	1 : 15.6
β-glob.	2.22	40.0	545.4	1 : 13.6
γ-glob.	1.48	15.8	286.3	1 : 18.1

Differences in Carbohydrate Content between Caseins from Cow's and Human Milk

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Important biochemical differences between human and cow's caseins have been described, *e. g.* in elementary composition, physicochemical properties and digestibility [1]. During preparation of human casein our attention was directed to the rather mucinous consistence of this protein in contrast to the bovine preparations. No investigation of carbohydrates in human casein has so far appeared in the literature. The present authors have therefore compared the content of hexose, hexosamine and sialic acid in human and cow's caseins.

The material was obtained from fresh, unpasteurized cow's milk and pooled frozen human milk. Bovine casein was prepared merely by acidifying the skimmed milk to pH 4.6 and treated according to conventional methods. Human casein was precipitated at pH 4.6 after diluting skimmed milk several times and further treated as the bovine casein. In no case reducing sugar was present in the casein preparations.

The caseins were investigated for hexoses by the orcinol method [2], hexosamine by Morgan-Elson's method [3] and sialic acid by Bial's reaction [4].

	% Hexose	% Hexosamine	% Sialic Acid
Cow's casein	0.23	0.19	0.45
Human »	1.98	1.32	0.75

The variance of the results between different samples from the same species was small. Thus, the carbohydrate content of human casein was found to be much higher than that of bovine casein. This difference was still more pronounced when the human casein was prepared from undiluted milk after dialysing against distilled water for several days.

The carbohydrate content of caseins from certain other species (*e. g.* sheep, goat and whale) and its correlation to hydrolysis with proteolytic enzymes will be discussed.

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Isolation of Sialic Acid from Brain Gangliosides

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It was from gangliosides that neuraminic acid was first isolated (Klenk) [1]. Using the same procedure he also isolated the acid from submaxillary mucin [2]. Blix, however, had more than 10 years earlier obtained from the same mucin an acid which was later named sialic acid [3]. The relation between the two acids was supposed to be that neuraminic acid was a deacetylated methoxy-derivative of sialic acid [4]. Thus, it seemed quite possible that sialic acid would also be the naturally occurring acid in gangliosides, and this the author has demonstrated to be the case.

Gangliosides were prepared by the cellulose column technique [5]. Hydrolysis was performed with 0.1 *N* H_2SO_4 for one hour at 90° C. The hydrolysate was poured into a cellophane bag and dialysed against distilled water changed twice a day. The dialysates were pooled, neutralised with barium hydroxide, and after filtration passed over Amberlite IR 120 (H) to remove excess Ba^{++}. The sialic acid was fixed on a Dowex 2 column (formate form) and eluted with increasing concentration of formic acid. Sialic acid was indicated in the eluates with Bial's reagents. Free sialic acid appeared when the normality of the formic acid in the eluate was 0.4—0.5. The fractions containing sialic acid were put together and evaporated *in vacuo*. Crystallization occurred in some instances already during evaporation.

The isolated substance, like sialic acids from mucin and serum (the latter isolated in the same way by the author), gave positive direct Ehrlich reaction (neuraminic acid: neg.). Ninhydrin reaction was negative (neuraminic acid: pos.).

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2. Klenk, E. und Lauenstein, K. *Ibid.* **291** (1952) 147.
3. Blix, G., Svennerholm, L. and Werner, I. *Acta Chem. Scand.* **6** (1952) 358.
4. Blix, G., Lindberg, E., Odin, L. and Werner, I. *Nature* **175** (1955) 340.
5. Svennerholm, L. *Acta Chem. Scand.* **8** (1954) 1108; *Nature (In press).*

Glutamine in the Biosynthesis of Mammalian Mucopolysaccharides

Harry Boström, Lennart Rodén and Anders Vestermark

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Boström and Månsson [1,2] studied the factors governing the incorporation of ^{35}S-labelled sulphate into the chondroitin sulphuric acid of surviving calf cartilage. It was found that small amounts of a liver homogenate added to the incubation medium greatly increased the ^{35}S incorporation [3]. The active principle (named sulphate exchange stimulating factor: S.E.S. factor) could be purified from calf liver by means of fractional precipitation of a boiled liver extract with mercuric acetate and subsequent preparative paper electrophoresis [4]. When subjected to paper chromatography, the electrophoretically purified fraction showed 7 ninhydrin-positive spots, one of which contained the whole S.E.S. activity. The chemical nature of the S.E.S. factor has not yet been determined, but strong evidence points towards the identity of the factor with glutamine [5]. Some facts pertinent to this supposition are presented below.

1) The S.E.S.-active ninhydrin-positive spot and L-glutamine have the same R_F value in the solvents used for two-dimensional paper chromatography (80 % ethanol in water; *sec*-butanol-90 % formic acid-water: 75+15+10 parts, respectively).

2) In chromatography on the cation exchange resin Dowex 50, L-glutamine and the S.E.S. factor have the same effluent volume, both when tested separately and in mixed chromatography. The recovery of glutamine is not quantitative; this also applies to the S.E.S. factor.

3) L-Glutamine has a five times greater S.E.S. activity per mg of dry substance than that of the electrophoretically purified S.E.S. factor from calf liver.

Glutamine could not be replaced by asparagine, aspartic acid, glutamic acid, glutathione, ammonium chloride or glutamic acid + ammonium chloride.

Lowther and Rogers [6] have shown that glutamine increases the quantity of bound glucosamine formed by streptococci, and Leloir and Cardini [7] have studied the role of glutamine in the biosynthesis of glucosamine in cell-free extracts of *Neurospora crassa*.

On the basis of the observations reported above, we can now assume that glutamine also plays an important part in the biosynthesis of animal mucopolysaccharides.

1. Boström, H. and Månsson, B. *J. Biol. Chem.* **196** (1952) 483.
2. Boström, H. and Månsson, B. *Arkiv Kemi* **6** (1953) 23.
3. Boström, H. and Månsson, B. *Acta Chem. Scand.* **7** (1953) 1014.
4. Boström, H., Jorpes, E., Månsson, B., Rodén, L. and Vestermark, A. *Arkiv Kemi* (1955). *In press.*
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Quantitative Determination of Bile Acids in Bile

Jan Sjövall

Department of Physiological Chemistry, University of Lund, Lund, Sweden

A method for the quantitative determination of mixtures of synthetic bile acids has recently been published [1]. The bile acids are separated by paper chromatography, eluted from the chromatograms and then determined by spectrophotometric assay in sulfuric acid.

This method has now been adapted for the determination of bile acids in bile. A suitable volume of bile, usually in the order of 10—20 µl, is applied on the starting line as a spot. The chromatogram is developed and the bile acids are localized as earlier described [1]. The spots are then cut out and eluted with ethanol. A corresponding filter paper blank is eluted in the same way. The bile acid content then can be determined as earlier described for synthetic bile acids. Other substances present in the bile do not interfere with the quantitative determination. A blank value which depends on the bile pigment concentration is satisfactorily corrected for by measuring the optical density of one bile acid sample in ethanol and another in 65 % sulfuric acid as described. The bile acid content can be calculated from the value obtained by subtracting the value in ethanol from that in the sulfuric acid. Added amounts of known bile acids are quantitatively recovered. Some results obtained by this method will be described.

1. Sjövall, J. *Arkiv Kemi* **8** (1955) 317.

The Mucopolysaccharides of *Nucleus Pulposus*

Sven Gardell

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

From sulphate and monosaccharide analysis it has been concluded that the mucopolysaccharides of human *nucleus pulposus* is chondrotinsulphuric acid and keratosulphate [1].

These two polysaccharides have now been isolated and identified. A dried preparation of *nucleus pulposus* was heat-coagulated and digested with glycerol extracts of pancreas and of intestinal mucosa [2] followed by precipitation with alcohol. The precipitate was extracted with phenol and the digestion and extraction procedure repeated once [1].

In this way a polysaccharide fraction with a ratio nitrogen/aminosugar of 1.5 was obtained. Glucosamine and galactosamine were both present [3] in a ratio of 0.8.

The fractionation of the purified material was most easily accomplished by precipitation with ethanol on top of a cellulose column followed by elution with ethanol in decreasing concentrations.

Two peaks were obtained, when the effluent was analysed with Dische's carbazol method. In the first peak appearing at about 35 % ethanol the aminosugar component was made up to 90 to 95 per cent of glucosamine. The second peak appeared at an alcohol concentration of 10—15 %, and 95 to 100 % of the aminosugar was galactosamine. Galactose was found only in the first peak.

Details of the fractionation procedure together with analysis and properties of the fractions are given.

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The Effect of pH on the Balance between Oxidation of Ascorbic Acid and Reduction of Dehydroascorbic Acid in Plant Tissue

Frithiof Alm and Martin Lundberg

Swedish Institute for Food Preservation Research, Göteborg, Sweden

A rapid oxidation of ascorbic acid in raw cabbage leaves when sprinkled with diluted acetic acid has been demonstrated [1]. We were able to show that this oxidation is brought about by the ascorbic acid oxidase of cabbage [2]. The same effect of acetic acid is obtained in a large number of fruits and vegetables containing strongly active ascorbic acid oxidizing enzymes [3].

Further experiments have shown that in raw cabbage reduction of dehydroascorbic acid (DHA) by reduced glutathione under anaerobic conditions is much more sensitive to lowering of pH than aerobic oxidation of ascorbic acid (AA). Thus, the effect of acetic acid may be explained by a stronger inhibition of reduction of DHA in an acidified cell as compared to oxidation of AA.

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2. Alm, F. *Intern. Z. Vitaminforsch.* **23** (1952) 459.
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A New Abnormal Fe-Hemochromogen as a Cause of Hereditary Cyanosis

Harald Hansen, Rudolf Jagenburg and Tom Reinand

Chemical Central Laboratory, Sahlgrens Hospital and Pediatric Clinic, Gothenburg, Sweden

The authors have investigated the blood from a family with hereditary non cardiac cyanosis affecting 5 of 7 members representing three generations.

The maximal oxygen combining power was 20 % less than for a normal blood with the same iron content. Absorption spectral curves of oxygenated blood as compared with oxyhemoglobin from normal blood with the same iron content showed neither differences within the ultraviolet range (240 mμ—400 mμ) nor within the infrared (750 mμ—1 000 mμ). In the visible range there was found increased absorption from 480 mμ—520 mμ and between 590 mμ—670 mμ with a maximum at 600, and decreased absorption between 530 mμ—585 mμ indicating the presence of an abnormal hemochromogen. The non-identity with methemoglobin (MHb) and sulfhemoglobin was evident from different absorption spectral curves and further differing from MHb by the absence of reaction with cyanide and fluoride,

as well as from failing response *in vivo* to methylene blue or ascorbic acid (reduction of MHb to ferrohemoglobin). The abnormal hemochromogen could not be transferred to MHb by ferricyanid, nor could it be reduced by hydrosulfite or transferred to COHb, as judged from absorption spectral curves, whereas it could be transformed quantitatively to acid hematin. The peroxidase activity of the blood was the same as for normal blood with the same iron content. No difference from normal hemoglobin in electrophoretic mobility (paper electrophoresis) was found at pH 8.6.

It is concluded that the abnormal hemochromogen assumingly is a Fe-chromogen with peroxidase activity but not able to combine with oxygen and responsible for a new form of hereditary cyanosis. The hemochromogen is called Norin-hemochromogen referring to the name of the cyanotic family.

Cholyl-CoA as an Intermediate in Taurocholic Acid Formation by Rat Liver Microsomes

Jon Bremer

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

We have previously shown that both the microsomic fraction and the particle-free supernatant of rat liver homogenates are required for the conjugation of cholic acid with taurine [1,2].

The mechanism of this conjugation has been subjected to further studies.

Rat liver microsomes were isolated as follows: The liver was homogenized in 4 parts 10 % sucrose. Nuclei and mitocondria were sedimented at 16 000 g for 15 min, the microsomes at 25 000 g for 110 min. The fraction was washed once.

The taurocholic acid formation was followed by means of ^{35}S-labelled taurine as previously described [1]. It appears from Table 1 that the particle free supernatant could be substituted by CoA and ATP. The conjugation was also stimulated by Mg^{++} (though not in the published experiment) and fluoride. Cysteine or glutathion was required when a crude CoA was used, but when a reduced, 70 % pure CoA was used, the effect of cysteine or glutathion was slightly inhibiting.

Table 1. Complete system: Phosphate buffer pH 7.4 0.022 M; *KF 0.215* M; *Cysteine 0.007* M; *CoA 0.001* M; *ATP 0.007* M; *Mg^{++} 0.001* M; *Sucrose 0.035* M; *Microsomes from approximately 500 mg liver per vessel.*

Experiment I 2 *μmoles taurine/vessel*
2 *μmoles cholic acid/vessel*
Total volume 1.5 ml/vessel

Experiment II 50 *μmoles hydroxylamine/vessel*
1 *μmole cholic acid/vessel*
Total volume 1.75 ml/vessel

All solutions were adjusted to pH 7.4. Gas phase air, Incubation time 120 min, temp. 37° C.

Factor *excluded* from the complete system	*Experiment I* Taurocholate formed μmoles/vessel		*Experiment II* Hydroxamic acid formed μmoles/vessel	
None	0.31	0.27	0.71	0.69
Cholic Acid	0	0	0.03	0.03
KF	0.12	0.12	0.42	0.41
ATP	0	0	0	0
CoA	0	0	0.14	0.07
Cysteine	0.34	0.35	0.85	0.82
Mg^{++}	0.41	0.36	0.74	0.73
Hydroxylamine	—	—	0	0.05

These findings make it probable that cholic acid is conjugated with taurine with cholyl-CoA as an intermediate. This was further supported by the formation of cholic hydroxamic acid when hydroxylamine was added to the incubation mixture instead of taurine.

The cholic hydroxamic acid formed was isolated by a modification of Eriksson and Sjövall's [3] quantitative paper chromatography for conjugated bile acids. A mixture of 1 part *n*-butanol and 2 parts petroleum spirit (b. p. above 120° C) was used as a moving phase. The hydroxamic acids spots were eluted with ethanol, the eluate was blown to dryness and the residue dissolved in conc. H_2SO_4. After 1 hour the density at 389 mμ was measured.

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By this method the formation of cholic hydroxamic acid was shown to be dependent on the presence of ATP, CoA and fluorid. As hydroxylamine represents a trapping agent for CoA-activated carboxyl groups, it is concluded that cholyl-CoA represents the "activated cholic acid" which conjugates with taurine.

The ^{35}S-labelled taurine was generously supplied by L. Eldjarn, Norsk Hydro's Institute for Cancer Research, Oslo.

This work has been supported by *The Norwegian Cancer Society*, Oslo.

1. Bremer, J. *Acta Chem. Scand.* **9** (1955) *In press.*
2. Bremer, J. and Gloor, U. *Acta Chem. Scand.* **9** (1955) *In press.*
3. Eriksson, S. and Sjövall, J. *Acta Chem. Scand.* **8** (1954) 1099.

Observations on Biosynthesis of Lecithins

Donald J. Hanahan* and Martin Rodbell

University of Washington, Department of Biochemistry, Seattle, Washington, U.S.A.

Studies have been carried out on the metabolism of lecithin and certain of its derivatives in the subcellular fractions of the liver cell. Although lecithin was apparently not oxidized and had no obvious effect on the isolated mitochondria, lysolecithin, glyceryl phosphorylcholine, phosphorylcholine and glycorophosphate plus choline had a marked stimulatory effect on the oxidation rate of the mitochondrial systems. One of the most active compounds in this stimulatory action was phosphoryl choline which was very actively incorporated as an intact unit into lecithin. The implications of these observations and possible synthetic routes for lecithin will be discussed.

* Temporary address: Department of Physiological Chemistry, University of Lund, Lund, Sweden.

The Metabolism of Cysteamine Sulphinic Acid (Hypotaurine) in Rats, Investigated by Means of Radioactive Sulphur (^{35}S)

L. Eldjarn and A. Sverdrup*

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

Cysteamine sulphinic acid is formed by the mammalian organism and is an intermediate in the degradation of cysteine [1]. The compound is formed by the decarboxylation of cysteine sulphinic acid [2] as well as by the oxidation of cysteamine and cystamine [3].

In order to study the metabolism of cysteamine sulphinic acid, the compound was synthesized and labeled with radioactive sulphur. ^{35}S-labeled cystamine was prepared as previously described [3]. Cysteamine sulphinic acid was prepared from cystamine according to the following dismutation reactions:

$$(HCl \cdot NH_2CH_2CH_2S)_2 + Hg^{++} \longrightarrow NH_2CH_2CH_2SOH + NH_2CH_2CH_2SHgCl$$

$$NH_2CH_2CH_2SOH \longrightarrow NH_2CH_2CH_2SO_2H + (NH_2CH_2CH_2S)_2$$

The mercaptide was precipitated by 10 % ethyl alcohol. When 8 equivalents of $HgSO_4$ was used, a yield of 23 % of cysteamine sulphinic acid admixed 9 % cystamine. was obtained. The cystamine was removed by adsorbtion on Dowex 50. The cysteamine sulphinic acid was crystallized from water-ethanol ether. M. p. 170° C.

One mg of ^{35}S-labeled cysteamine sulphinic acid was administered to male rats (250 g). After fractionation of the urine [3] the greater part of the radioactivity was recovered as sulphate and as taurine. Most probable the metabolism of cysteamine sulphinic acid in the rat organism is confined to these two reactions. Taurine is known not to be converted to sulphate by the rat organism. These findings should provide good opportunity to study the mechanism of sulphate formation in mammals by means of cysteamine sulphinic acid labeled with radioactive sulphur.

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On the Sulphate Metabolism in the Sulphocerebrosides of the Brain

Åke Holmgård

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

In connexion with Boström's work on the sulphate exchange in the chondroitin sulphuric acid of cartilage, investigations have been made at our Institute of whether a similar exchange of sulphate takes place in heparin and in the cerebroside sulphuric acid of the central nervous system.

It was shown in a study by Magnusson and Larsson [1] that radioactive sulphate is incorporated in the heparin of mastocytomas in the dog. This was also found to apply to cerebroside sulphuric acid.

The sulphur uptake in the central nervous system has been studied by Dziewiatkowski [2] in the rat, using oral doses of sodium sulphide containing ^{35}S. He recovered 0.02 % of the dose given in the brain. The same experiments have been made in the rabbit, with similar results.

Boström and Odeblad [3] used the autoradiographic technique to demonstrate the sulphate uptake in the brain; they found the highest concentration in the grey matter.

In order to ascertain whether radioactive sulphate is incorporated in the sulpholipids of the brain, 4 rabbits were each given an intramuscular injection of 3 mC of carrier-free sodium sulphate containing ^{35}S. The animals were killed 108 hours later. The sulpholipid was isolated according to Blix [4], but it was not possible to perform the last stage, since the basic material was insufficient. To eliminate inorganic sulphate, the sulpholipid was passed at 42° C through an anion exchange column (Dowex 2). The activity was determined in a surface of 1.58 cm^2 and a layer of infinite thickness. The results are shown in Table 1.

Table 1.

	Weight g	Counts/ min.
Acetone-dried brain	5.06	110
Protagon	0.765	222
Sulpholipid	0.010	998

The same experiment was made in the rat, with similar results.

In order to rule out the possibility that the activity was due to contamination by chondroitin sulphuric acid, the following experiment was made. To 1.21 g of cattle protagon was added 0.91 g of ^{35}S-labelled chondroitin sulphuric acid, so that an activity of 527 counts/min. was obtained. The sulpholipid was then isolated from the mixture. It had no radioactivity.

It is concluded that an exchange has taken place between the sulphate group of the brain sulpholipid and the labelled free sulphate.

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Purification and Chemical Properties of Enterokinase

Ikuo Yamashina

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Enterokinase, an enzyme which accelerates the transformation of trypsinogen to trypsin, was purified as follows.

Duodenal fluid contents of swine was digested by proteolytic enzymes, followed by ammonium sulfate fractionation under pH control. The crude preparation thus obtained was submitted to the fractionation by organic solvents. Ethanol fractionation at pH 4.5 in the presence of 5 % calcium acetate and acetone fractionation at pH 5.5—5.9 in the presence of zinc were repeatedly applied. Finally, a highly active preparation (2 600 units per mg) which is 65 times more active than Kunitz's preparation in 1939 was obtained with the yield of 20 %. This preparation is fairly pure, in electrophoresis and ultracentrifugation. The isoelectric point is at around pH 4.0. The analyses showed that it contains a high content of carbohydrate (*ca.* 30 %), aminosugar being 13 % (glucosamine 10 % and galactosamine 3 %) and neutral sugars 15 % (fucose 4 %, mannose 3 %, and galactose 8 %) based on chromatographic methods.

The carbohydrate content increased following the increase of the activity during the purification.

Synthesis of Phospho-Peptides. O-Phospho-DL-Seryl-Glycine

Georg Fölsch

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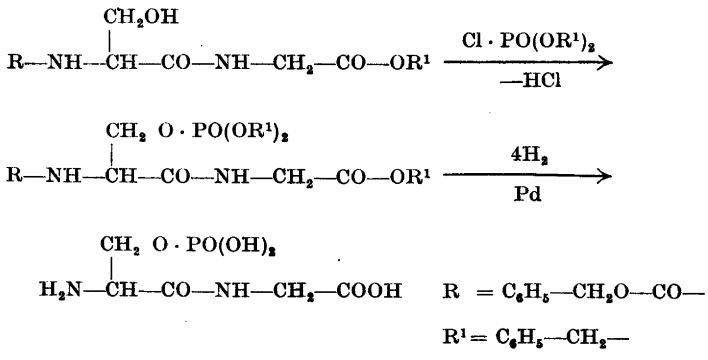
During recent years it has been suggested that phosphorylated peptides are of great biological significance [1-3]. A more detailed study of their properties depends partly upon the possibility of synthesising model peptides similar to the naturally occurring ones, *i. e.* peptides containing serine or threonine with phosphorylated hydroxyl groups.

Many investigations have been made on the phosphorylation of amino acids [4-6] and, also, peptides and peptide derivatives [7-9]. Although difficulties often appeared when preparing N-phospho- and also O-phospho-amino acids [4-6], Posternak and Grafl [9] succeeded in synthesising four peptides, which besides glycine also contained tyrosin with the phenolic hydroxyl group phosphorylated. No synthesis of phosphopeptides containing the only two O-phosphoamino acids isolated from natural sources, O-phosphoserine and O-phosphothreonine, has, however, been reported. We have, therefore, started to synthesise such peptides and now report a synthesis of O-phospho-DL-seryl-glycine. This compound has been obtained by phosphorylation of N-benzyloxycarbonyl-DL-seryl-glycine benzyl ester (prepared by coupling benzyloxycarbonyl-DL-serin azide with glycine benzyl ester) with dibenzyl phosphoryl chloride [10] at low temperature in pyridin solution, followed by removal of the benzyloxycarbonyl- and the three benzylester-groups from the reaction product by hydrogenation in presence of palladium catalyst:



The crude phosphopeptide (20 % yield) was further purified by column chromatography on Dowex 50. M. p. 178°. On paper chromatograms (phenol-water 80:20) the phosphopeptide gives colour both with ninhydrin (brown violet) and with phosphate reagent. It has an R_F-value of 0.13 when O-phosphoserine on same chromatogram has $R_F = 0.07$. Hydrolysis (6 *N* HCl, 20 h, 100°) gives serine, glycine and phosphoric acid as shown by paper chromatography. (Found: N 11.3; P 13,3. Calc. for $C_5H_{11}O_7N_2P$: N 11.6; P 12.8).

By the same procedure we are now preparing phosphopeptides containing L-phosphoserine. Further details will be published later.

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Purified Secretin and Pancreozymin

Erik Jorpes and Viktor Mutt

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For a number of years we have been engaged in work on the purification of the gastrointestinal hormones gastrin, secretin, pancreozymin and cholecystokinin. The impetus to this was given by repeated requests from clinical workers for these substances, the pharmaceutical houses being unable to provide them. From the classical work of Hammarsten, Ågren and Lagerlöf in Sweden and of Ivy and coworkers in the USA it is well known that secretin can be used for diagnostic purposes. The pancreozymin of Harper and Raper would, if available in purified form, evidently be a valuable supplement to secretin.

It proved possible to prepare a non-toxic secretin with an activity of 1 000 cat units per mg. This activity, assayed in the cat, was checked clinically [1]. The activity was surprisingly high in view of the fact that the crystalline secretin picrolonates reported in the literature, and claimed to represent the pure secretin [2] had an activity of 250 c.u. per mg.

Further work showed that the preparations assaying 1 000 c.u. per mg were still very crude. It was in fact possible to prepare secretin with an activity of 25 000 c.u. per mg [3]. The method for preparing this very active material is not yet worked out in detail and the yield of the material is poor. Preparations with an activity of 10 000 c.u. per mg can, however, be obtained routinely and have also been tested clinically.

We have applied isoelectric separations and fractionation with organic solvents as well as ion-exchange. In the latter the use of non-cross-linked naturally occurring acids and bases has been preferred instead of the various commercially available resins. The acids used have been polygalacturonic, polymannuronic and stearic, the bases stearylamine and deacetylated chitin.

For the purification of pancreozymin similar methods have been applied. We have prepared material which can be used clinically. A definite increase in output of pancreatic enzymes as well as a contraction of the gall bladder can be observed after the injection of 1 mg of this substance in an adult person.

By means of these purified hormones we have been able to confirm the view expressed by Pavlov long ago, that there are two mechanisms regulating the external pancreatic secretion, one controlling the secretion of water and bicarbonate and the other the secretion of enzymes.

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Quasi-racemic Compounds between Optically Active Positional Isomers of Methyl-substituted Octadecanoic Acids

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A pair of optical antipodes often crystallise together to form a racemic compound consisting of equal amounts of the two components. If one of the antipodes is subjected to a small change in the molecular structure it may still form a compound with the unaltered antipode. Such compounds have been called quasi-racemic by Fredga [1], who has made extensive use of them for the determination of the steric configuration of optically active molecules.

It has now been found that such a quasi-racemic compound is formed between (+)-9-D-methyloctadecanoic acid and (+)-10-L-methyloctadecanoic acid. The antipodes of both 9- and 10-methyloctadecanoic acid melt at 12.5—12.9°. 9-D,L-Methyl-octadecanoic acid melts at 38.7—39.2° whereas the racemic compound between the antipodes of 10-methyloctadecanoic acid is dimorphous with melting-points of 20.9—21.6° and 25.8—26.1° respectively [2]. An equimolecular mixture of (+)-9-D-methyloctadecanoic acid and (+)-10-L-methyloctadecanoic acid (antipode of tuberculostearic acid) melts at 18.6—18.7°. The corresponding mixture of (+)-9-D-methyloctadecanoic acid and (—)-10-D-methyloctadecanoic acid melts at about 5°.

Details will be published later.

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The Metabolism of Phosphoproteins in *Lactobacillus casei*

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John Glomset

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The incorporation of radioactive inorganic phosphate into the phosphoprotein fraction of *L. casei* has been compared with the phosphorus uptake of nucleotide and nucleic acid fractions during the transition from lag to log phase.

Aliquots of a 90 liter bacteria culture were taken at varying intervals subsequent to inoculation of the ^{32}P-containing medium. Bacteria were prepared from the aliquots as described previously [1]. Fractionation was carried out by a modified Schneider procedure. The 10 and 5 % TCA extracts were extracted with ether and run on Dowex 1, formate columns [2], the nucleic acid fraction after partial hydrolysis with 0.1 *N* KOH, 100°, 2 hours. The alkali-stable fraction from the 5 % TCA extract was hydrolysed enzymatically with desoxyribonuclease and snake venom diesterase and then fractionated on a Dowex 1 column. The "rest protein" was treated as described previously [3]. Table 1 shows the uptake of radioactive phosphorus into the major phosphorus-containing, ninhydrin-positive component of the "rest protein" partial acid hydrolysate compared with the number of bacteria and the number of counts per μg dry bacteria.

Table 1.

Time after inoculation in min.	Bacterial count Number/ml $\times 10^9$	Dry bacteria *cpm*/μg	Component of the rest protein, *cpm*/μg P
0	48	—	—
30	51	96	320
40	52	82	210
50	55	102	700
60	55	147	1 310
70	56	143	1 250
80	74	199	2 350

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Differences between Phosphoproteins of Animal and Bacterial Origin

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Carl-Henric de Verdier and
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The occurrence of phosphoserine and phosphothreonine in partial hydrolysates of the "Schneider protein" fractions of rat tissues has previously been reported [1]. We now have succeeded to isolate these two compounds in crystalline form from sheep liver tissue. The first one has already been isolated from calf liver tissue [2]. The same method of preparation was used now. The compounds were identified by X-ray powder diagrams. Sufficient amounts of serinephosphate permitted micro chemical analysis. (Found: C 19.53; H 4.52; N 7.73; P 16.75. Calc. for $C_3H_8O_6NP$: C 19.46; H 4.32; N 7.57; P 16.76).

In earlier communications we also reported the presence of phosphoserine in partial hydrolysates of protein from *Lactobacillus casei* [3,4]. These hydrolysates contain in addition a number of other ninhydrin-positive, phosphorylated compounds, but so far it has not been possible to demonstrate the presence of threoninephosphate. One of these compounds is present in much greater quantities than is phosphoserine. On complete hydrolysis it gives rise to a ninhydrin-positive compound which does not correspond in position to either serine or threonine on paper chromatograms. The possible identity of the compound is discussed. Details of these experiments will be published later.

1. Ågren, G., de Verdier, C-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1570.
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The Relation between Cholecystokinin and Substance P

Eric H. Hultman

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

The fact that a hormonal mechanism is responsible for contraction and emptying of the gallbladder was first demonstrated by Ivy (1928) [1], who named the hormone cholecystokinin. The methods for biological determinations of the hormone have varied since then, from pressure measurements on anaesthetized animals to intestinal bath experiments on isolated gallbladder. In experiments on isolated organs, cholecystokinin preparations have proved to contract not only the gallbladder, but also the isolated intestine of the guinea-pig. As late as 1953 and 1954, Gershbein *et al.*[2,3] stated that the guinea-pig intestine is as well suited as the gallbladder for a study of the cholecystokinin effect. The relation to substance P, studied by von Euler and Gaddum [4] and by Pernow [5], has been discussed, and the question whether these two substances are identical has been raised.

In experiments on the purification of cholecystokinin preparations, it was found that the intestinal effect could be entirely removed (Hultman 1955) [6], and that the factor in the crude preparations which produced intestinal contractions *in vitro* is, in all probability, substance P. Purified cholecystokinin preparations proved to have an effect *in vitro* on the guinea-pig gallbladder but not on the intestine, whereas substance P preparations were active on the guinea-pig intestine but not on the gallbladder (Table 1).

Table 1. Intestinal bath experiment (10 ml intestinal cuvettes, readings in mm on a scale).

		Intestine	Gallbladder
Substance P	3.3 units	10	0
H 289	0.05 mg	0	10
Substance P	3.3 units	9	0
H 289	0.05 mg	0	10
Substance P	6.6 units	10	0
H 282	0.07 mg	10	0
Substance P	1.2 units	2	0

Substance P obtained from Dr. Pernow. H. 282: a fraction obtained from the crude cholecystokinin. H 289: purified cholecystokinin (after removal of H 282).

The strongest preparations of cholecystokinin produced maximal contraction of the isolated guinea-pig gallbladder in doses of 1 μg/ml of fluid in the bath. On some sensitive gallbladders, a good effect was obtained with 0.5—0.1 μg/ml.

Table 2. Intestinal bath experiments as above (10 ml cuvettes).

		Gallbladder
P.S. 117	0.05 mg	10
H 271	0.005 mg	10
H 272	0.025 mg	12
Acetylcholine	0.001 mg	20

P.S. 117, H. 271, H 272: different purified cholecystokinin preparations.

Intravenous administration of the preparations produced a powerful secretion of bile in the anaesthetized cat.

In paper electrophoresis the purest substance showed an isoelectric area around pH 7. The preparations have, in addition, a strong effect on the secretion of pancreatic enzymes.

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4. v. Euler, U. S. and Gaddum, J. H. *J. Physiol.* **72** (1931) 74.
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On the Rate of Excretion of Bile Acids in the Rat

Sven Lindstedt and Arne Norman

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Rats were given [24-^{14}C]-labelled bile acids intraperitoneally. The excretion of activity in the faeces was followed. Practically all of the isotope appeared in the faeces [1]. From the rate of excretion the half life of a bile acid molecule was estimated to be 2—3 days.

A certain amount of bile acid is thus lost during each enterohepatic circulation. One of the factors influencing the magnitude of this loss might be the intestinal microorganisms.

In order to study this, the rate of excretion was followed in rats in which the intestinal flora had been reduced by treatment with terramycin and phthalylsulfonazonum. In these rats the rate of excretion was much slower than in normal animals.

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Demonstration of a Thermostable Cofactor of Oxidative Phosphorylation in Rat Liver Mitochondria

Lars Ernster and Hans Löw

Wenner-Gren's Institute, Stockholm, Sweden

It has previously been reported that addition of adenosine triphosphate (ATP) and Mn^{++} to mitochondrial suspensions pretreated in the presence of 0.5 m*M* Ca^{++} and 4 m*M* Mg^{++} for 5 minutes at 30° C induces a reconstruction of the phosphorylations accompanying the aerobic oxidation of succinate [1]. Subsequently, these effects could be studied in greater detail with mitochondrial systems aged in Mg^{++} — and Ca^{++} — free media [2]. The yield of reconstructed phosphorylation in these systems shows an inverse relationship to the time of aging, and its fall can be greatly enhanced by the presence of Ca^{++} in the Mg^{++}-free preincubation medium. Recentrifugation studies of the aged suspensions have revealed that, during aging, a factor is released from the mitochondria, which is involved in the phosphorylative mechanism [3]. This factor has now been shown to be stabilized by heating the preincubation medium, obtained after recentrifugation, to 100° C for 2 minutes. Furthermore, the presence of the factor could be demonstrated in boiled extracts of freshly prepared mitochondria. Experimental data bearing on this are shown in Table 1.

Mitochondria from one rat liver were suspended in 30 ml of a buffer solution (pH 7.5) containing KCl, orthophosphate, adenylic acid, glucose and sucrose (in concentrations specified previously [2]). After removal of a sample serving as control (incubated immediately in the presence of succinate, hexokinase and Mg^{++}), the suspension was warmed to 30° C and supplemented with hexokinase and 0.5 m*M* Ca^{++}. After 5 minutes of preincubation under shaking, the suspension was cooled to 0° C, divided into four parts and each part recentrifuged for 5 minutes at 5 000 *g* in the high-speed attachement of an International Refrigerated Centrifuge. The four mitochondrial pellets (*a*—*d*) were resuspended, to a final volume equal to that before centrifugation, in *a*) a new incubation mixture of the same composition as before; *b*) its own supernatant; *c*) the supernatant heated for 2 minutes in a boiling water bath (the slight precipitate formed on heating was removed by centrifugation); and *d*) in an extract of freshly prepared mitochondria, obtained by heating a suspension (of the same composition as the original one, but twice as concentrated with respect to mitochondria) for 2 minutes in a boiling water bath, and centrifugation as above. From each of the four suspensions two aliquots of 1.5 ml were incubated in Warburg vessels, one with succinate alone, and a second one with succinate, ATP and Mn^{++}. Each vessel was furthermore supplemented with Mg^{++} and a suitable excess of hexokinase. Respiration and phosphorylation were measured for a period of 20 minutes.

Table 1. Oxydative phosphorylation in recentrifuged mitochondria aged in the presence of Ca^{++}.

Each Warburg vessel contained: KCl, 150 μmoles; orthophosphate, 50 μmoles; adenylic acid, 4.3 μmoles; glucose, 60 μmoles; sucrose, 125 μmoles; succinate, 30 μmoles; Mg^{++}, 4 μmoles; hexokinase, *ca.* 10 fold excess[2]; Ca^{++} (in pretreated systems), 1 μmole. Additions (where indicated): ATP 1.5 μmoles; Mn, 1 μmole. Final volume, 2.0 ml. Gas phase, air. Temp, 30° C. Time of incubation, 20 min.

	Additions	Respiration (μatoms oxygen)	Phosphorylation (μmoles phosphate)
Untreated system	—	14.9	27.0
Pretreated system resuspended in:			
a) new incubation mixture	—	18.0	1.0
	ATP, Mn^{++}	16.7	0.5
b) supernatant	—	16.6	2.1
	ATP, Mn^{++}	14.0	1.3
c) boiled supernatant	—	18.3	1.0
	ATP, Mn^{++}	15.9	20.0
d) boiled extract of fresh mitochondria	—	14.5	1.4
	ATP, Mn^{++}	18.0	23.2

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d) boiled extract of fresh mitochondria	—	14.5	1.4
	ATP, Mn^{++}	18.0	23.2

As seen in Table I, under the prevailing experimental conditions, no phosphorylation can be reconstructed by ATP and Mn^{++} in the system resuspended in new incubation medium, or in the original supernatant. When boiled supernatant, or a boiled extract of fresh mitochondria is used as a suspending medium, reconstruction of phosphorylation again becomes possible. This indicates that the falling yield of the reconstructed phosphorylation, previously observed to occur on prolonged preincubation in Mg^{++}-free medium, may be accounted for by a destruction, probably enzymic in nature, of the released factor.

1. Ernster, L., Lindberg, O. and Löw, H. *Nature* **175** (1955) 168.
2. Ernster, L. and Löw, H. *Exptl. Cell Research* **Suppl. 3** (1955) 133.
3. Ernster, L., Löw, H., Nordenbrand, K. and Ernster, B. *Exptl. Cell Research* **9** (1955). *In press.*

Rapid High Precision Conductivity Recorder

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Olof Mellander

Department of Medical Biochemistry, University of Gothenburg, Gothenburg, Sweden

For studies of enzymatic degradation of peptides and proteins and micelle formation in bile salt solutions as well as for conductometric titrations we needed a conductivity recorder of high precision and rapid response, stable enough to follow small resistance changes over long periods of time. Several automatic recording devices have been described in the literature (*cf.* Refs.[1,2]). Most of these have been constructed for use as detectors in chromatographic analysis and none appeared to have the desired combination of high sensitivity, speed, and stability needed for our purposes. This also applied to commercially available instruments.

We have, therefore, converted a Shedlovsky precision bridge[3] into a high precision resistance recorder in the following manner.

The bridge is fed from a 1 000 c/s r.c. oscillator. The detector signal from the bridge is amplified by a three stage logaritmic amplifier which prevents overloading of the recording system for large unbalance signals. The amplified signal is rectified by a phase sensitive homodyne detector of the type described by Kinell[4]. The filtered output from the homodyne is fed to a Speedomax G recorder. To the potentiometer shaft of the recorder is mechanically coupled a linear high precision potentiometer which forms parts of two arms of the bridge, the moving contact being connected to the detector amplifier. A signal from the bridge makes the recorder pen move until the resistance balance of the bridge has been restored through the potentiometer attached to the pen movement. In order to prevent hunting in the servo system an adjustable velocity feed back to the recorder amplifier is provided from a D.C. generator geared to a balance motor of the recorder. Through a variable shunt across the potentiometer coupled to the pen, the resistance change for full scale response of the recorder may be adjusted over a 1:200 range (*e. g.* 10:2000 Ω). The speed of response is practically that of the recorder (4.5 secs. in the Speedomax G used). The lock-in amplifier detector system used gives a very low noise level which makes the recorder detect smaller resistance changes than the human ear using head phones. A sensitivity of 2 parts per 100 000 has been reached with no detectable noise in the recordings. Applications will be described.

1. Drake, B. *Arkiv Kemi* **4** (1952) 401.
2. de Verdier, C.-H., and Sjöberg, C. I. *Acta Chem. Scand.* **8** (1954) 1161.
3. Shedlovsky, T. *J. Am. Chem. Soc.* **52** (1930) 1793.
4. Kinell, P.-O. *A spectrophotometric study of polymethyl methacrylate*, Diss. Uppsala 1953.

Studies on Pancreatic Lipase

Bengt Borgström

Department of Physiological Chemistry, University of Lund, Lund, Sweden

A simple and rapid method for the determination of pancreatic lipase activity is described. It is based on the rate of clearing of a triolein emulsion by the lipase.

Using this assay method lipase from human and rat pancreatic juice has been purified by salt precipitation and electrophoresis on starch block according to Kunkel. The purest fractions so far obtained have an activity approximately 1 500 times that of extracts from acetone powder of pancreatic glands (calculated per mg of protein) (viokase).

No evidence of any esterase activity in human or rat pancreatic juice has been found.

The effect of bile acids, fatty acids and some other substances on the rate of hydrolysis of triolein by purified lipase will be described.

As seen in Table I, under the prevailing experimental conditions, no phosphorylation can be reconstructed by ATP and Mn^{++} in the system resuspended in new incubation medium, or in the original supernatant. When boiled supernatant, or a boiled extract of fresh mitochondria is used as a suspending medium, reconstruction of phosphorylation again becomes possible. This indicates that the falling yield of the reconstructed phosphorylation, previously observed to occur on prolonged preincubation in Mg^{++}-free medium, may be accounted for by a destruction, probably enzymic in nature, of the released factor.

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No evidence of any esterase activity in human or rat pancreatic juice has been found.

The effect of bile acids, fatty acids and some other substances on the rate of hydrolysis of triolein by purified lipase will be described.

The Assay of Vitamin B_{12} in Blood Serum and Urine

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Of the microorganisms at present in use for vitamin B_{12}-assay, the following have been tested:

1. *Euglena gracilis var. bacillaris.* The method described by Ross [1] has been somewhat modified. The inoculum is washed and the chlorophyll determined photometrically after 10 days incubation.

2. *Escherichia coli 113-3.* The medium described by Burkholder [2] has been used. The glucose is autoclaved and added separately and the incubation time is 24 hours.

3. *Ochromonas malhamensis.* The method described by Ford [3] has been used with an incubation time of 5 days.

Specificity. In the presence of methionine, *E. coli* does not require vitamin B_{12} as a growth factor. Only exceptionally the serum-concentration of methionine is so high as to interfere with the vitamin B_{12}-assay. Some of the vitamin B_{12}-like compounds, obtained from Dr. Ford, have been tested for growth activity. The results, which are given in Table 1, show the high specificity of *Ochromonas malhamensis* compared to the others.

Table 1.

	Eugl. grac. var. bacill.	*E. coli* 113—3	*Ochrom. malham.*
Cryst. vitamin B_{12} (cyanocobalamin)	100	100	100
Factor A	77	50	0
Factor B	13	26	0
Pseudovitamin B_{12}	27	5	0
Vitamin B_{12} III (Bernhauer)	91	79	32

Sensitivity. A vitamin B_{12}-conc. of 0.5 $\mu\mu$g/ml is sufficient for stimulating growth of *Euglena gracilis.* The corresponding values for *E. coli* and *Ochromonas malhamensis* are 5 and 2.5 $\mu\mu$g/ml, respectively. Because of the need of a method of maximum sensitivity for the assay of vitamin B_{12} in serum and urine, *Euglena gracilis* has been used in this investigation.

Results. The normal range changes with age from relatively high values at birth to lower conc. during the first years. During youth high values are found and in senescence relatively low conc. compared to the serum-conc. of vitamin B_{12} in middle age. Very low conc. of vitamin B_{12} in serum and urine have been found in pernicious anemia in relapse. Extremely high values have been demonstrated in chronic myelocytic leucemia. Normal to moderately increased values have been found in cases of polycythemia, osteosclerotic anemia, Hodgkin's disease, cancer metasthases of the bone-marrow, hepatic cirrhosis and diabetes mellitus. In acute leucemia, chronic lymfocytic leucemia and myeloma normal values have been found. The excretion in the urine of vitamin B_{12} is normal in all cases except pernicious anemia. The vitamin B_{12}-assay has been shown to be of great value in establishing the diagnosis of pernicious anemia in relapse at a very early stage and especially of subacute combined degeneration of the cord without characteristic findings in the blood and bone-marrow.

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2. Burkholder, P. R. *Science* **114** (1951) 459.
3. Ford, J. E. *Brit. J. Nutrition* **7** (1953) 299.

Hemoglobin Formation in the Rat Liver Nucleus

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The nucleus of the liver cell contains a large part of the iron content of the cell. A few hours after intraperitoneal injection of ^{59}Fe a considerable part of this iron is found in the liver cell nucleus. Part of this iron is due to the presence of hemoglobin iron.

The presence of hemoglobin iron in the nucleus is not due to contamination with hemoglobin of the circulating blood as the specific activity of the latter is appreciably lower than that of the hemoglobin present in the nucleus.

Chromatographic Separation of Serum Lipoproteins

L. A. Carlson

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The presence in blood serum of different types of lipoproteins has been well established during recent years in particular by means of Cohn-fractionation, electrophoresis and ultracentrifugation.

Chromatographic methods, however, have as yet not been applied to the study of serum lipoproteins. Incidentally it was observed in this laboratory that the serum lipoproteins were adsorbed onto glass beads. Furthermore it was found that they could be eluted from columns of glass beads by means of alkaline buffers. Then the possibility to separate chromatographically the different types of lipoproteins was investigated.

Serum lipoproteins were adsorbed onto columns of glass beads and eluted with a continuous pH-gradient according to the device of Bock and Ling [1]. In this way it has been possible to separate three different groups of lipoproteins in human serum: a) A lipoprotein containing phospholipids but no cholesterol; b) Lipoproteins with a cholesterol-phospholipid ratio below 1; c) Lipoproteins with a cholesterol-phospholipid ratio above 1.

1. Bock, R. M. and Ling, N-S. *Anal. Chem.* **26** (1954) 1543.

Synthesis and Metabolism of 2,2-Dimethylnonadecanoic Acid

N. Tryding and G. Westöö

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2,2-Dimethyl[1-^{14}C]nonadecanoic acid has been prepared by a Kolbe electrolysis of heptadecanoic acid and 3-methoxy[^{14}C]carbonyl-3-methylbutyric acid. The intestinal absorption and metabolism of this acid has been studied in the rat. The main metabolic end products were 2,2-dimethylglutaric acid and 2,2-dimethylpimelic acid, which were recovered in the urine. These products were identical with corresponding synthetic compounds. The results will be discussed in relation to earlier results on the metabolism of branched chain fatty acids.

The Anticoagulant Effect of Brain Gangliosides

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Anticoagulants of a lipid nature have been described from several animal sources, especially from brains. Most authors have attributed the inhibition to the inositol-phosphatide fraction [1,2]. However, no definite proof of its chemical identity has been presented.

One of us (K. K.-B.) has found that surface active agents even in rather low concentrations give a pronounced inhibitory effect on the first phase of blood coagulation. During his work with brain gangliosides Svennerholm observed that these substances have great surface activity. We therefore started an investigation of their effect on blood coagulation.

Gangliosides were prepared by the cellulose column technique [3]. By this method the gangliosides were separated in two fractions, ganglioside 1 containing 8 % hexosamine (G-1), and ganglioside 2 containing 2 % hexosamine (G-2).

To test the inhibition in the clotting system we used a modified thromboplastin generation method [4]. The lipids to be tested were dissolved in physiological saline and added to a system containing antihaemophilic factors A and B, platelet lipid factor and $CaCl_2$. After incubation for exactly 5 minutes an aliquot of the sample was tested for thromboplastic activity.

	G-1 4 μg	G-2 6 μg	Physiol. saline
Clotting time in sec.	65	35	25

As seen from the results chondrosamine containing ganglioside is a powerfull inhibitor of the first phase of the blood coagulation.

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2. Overman, R. S. *Ibid.* pp. 29—50.
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4. Biggs, R. *J. Clin. Pathol.* **6** (1953) 23.

Amino Acids and Peptides in Infant Urine

Rudolf Jagenburg

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The figures given in the literature for the normal excretion of amino acids in urine vary a lot, and refer mostly to urine from adults [1,2]. The following is a preliminary report of an investigation of the amino acid and peptide composition in urines from infants including prematures.

Method. Two dimensional paper chromatography. First solvent: butanol, acetic acid, water. Second solvent: phenol, water, ammonia. Desalted urine (during this process all taurine is lost) corresponding to 400 or 600 μg of total nitrogen was put on each paper.

Samples of urine from 40 infants, age 3 weeks to one year, have been analysed. Thirty-two spots occurred relatively frequently. Eighteen spots, arranged according to falling approximate amino acid content, have been identified as glycine, histidine+arginine (only small amounts), alanine, glutamine, serine, tyrosine, glutamic acid, threonine, asparagine, lysine, valine+tryptophane+methionine, cystine, α-amino-n-buturic acid, aspartic acid, phenylalanine, *iso*leucine+leucine, proline, hydroxyproline.

Three frequently occurring spots show the same positions on the chromatogram as have been reported in the literature [3-5] for β-amino *iso*butyric acid, γ-amino n-buturic acid and α-amino adipic acid. Two other but fainter spots show the same positions as a synthetic mixture of 1- and 3-methylhistidine.

Of the remaining 9 spots two, that seem to be peptides, are of special interest. They give the same typically yellow colour with ninhydrin as proline, but differ markedly in their positions on the chromatogram. $R_{proline}$ values in butanol/phenol: 0.85/0.80, 0.81/1.00.

The first of these substances, which sometimes occurs in relatively great amounts, was purified, and after hydrolysis contained proline, glycine, alanine and glutamic acid, the colour of the proline and glycine spots being stronger than that of the others. The nature of the remaining unidentified spots is under investigation.

As a rule the amino acid and peptide patterns from different individuals are rather constant, variations however being observed. Proline and hydroxyproline have only been found in urine from premature babies. The composition of food (human milk — cow's milk) seems not to have any influence on the amino acid and peptide excretion; at any rate no difference can be detected in the chromatograms.

Ten urines from adults have also been analysed. The two yellow spots have not so far been found in any urine from adults.

1. Stein, H. *J. Biol. Chem.* **201** (1953) 45.
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3. Crumpler, H., Dent, C., Harris, H. and Westall, R. *Nature* **167** (1951) 307.
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Metabolite Analogues Containing a Geminal Dimethyl Group

Åke Jönsson

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In the course of an investigation originally devoted to the synthesis of compounds with auxin (plant growth hormone) activity it was observed that synthetic auxins of the arylacetic, aryloxyacetic and 3-indoleacetic acid type were quite generally converted into antiauxins (auxin antagonists) by the introduction of a *gem*-dimethyl group at the methylene carbon atom. This fact encouraged us to prepare similar "branched" analogues of some animal metabolites such as certain amino acids, hormones and vitamines in order to study whether this structural alteration of the metabolites might produce interesting changes in the physiological activities of the compounds. So far these studies have resulted in synthetic methods for the preparation of analogues of aspartic acid, phenylalanine, tyrosine, thyroxine, histamine, pantothenic acid, and tryptamine, and the physiology and pharmacology of these compounds is now being studied in various institutes. Investigations now in progress are devoted to the analogues of aliphatic amino acids with the ultimate goal of preparing folic acid analogues containing "branched" amino acid moieties. The synthetic methods for these types of compounds as well as the test results obtained so far will be discussed.

On the Reactivity of Thiol Groups in Ox Heart Lactic Dehydrogenase

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Lactic dehydrogenase (LDH) is not affected by several common sulfhydryl reagents and is just slowly inactivated by *p*-chloromercuribenzoate (PCMB) [1]. In contrast, silver and mercuri ions have been found to inactivate the enzyme very rapidly. The inhibition was counteracted by cysteine. Amperometric titration of the native LDH with mercuri chloride showed the presence of 7—8 thiol groups.

Both the rate and the extent of the reaction with PCMB were increased after acetylation of LDH with acetic anhydride and after denaturation of the protein with lauryl sulfate.

Iodine was found rapidly to oxidize essential thiol groups. The inactivation was reversed by cysteine. No substitution of tyrosine took place at pH 7. In contrast to iodine, the strong oxidizing agent periodate inactivated LDH very slowly.

The facts that mercuri, silver and iodine were found to oxidize essential thiol groups rapidly, whereas PCMB and periodate caused much slower inhibition, could be due to steric hindrances of the larger molecules. Since the substrates and the coenzyme are comparatively large molecules, thiol groups may not be of direct importance in the binding of coenzyme or substrate in LDH.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 1049—1056

Evidence for the Presence of the —OH···OC-Link in Collagen from its Fixation of Non-ionic Chromium Complexes

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Data on the fixation of various chromium complexes, particularly nonionic sulphito-chromium sulphates by intact collagen, on the one hand, and by denatured and otherwise modified collagens, particularly hide protein of various degrees of acetylation (O- and N-acetylated), on the other, are in harmony with the postulate that a considerable part of the hydroxy groups of mammalian collagen are internally compensated by stable hydrogen bonds with the oxygen atoms of the keto-imide groups of adjacent chains.

The presence of an interchain crosslink of the hydrogen bond type, between the hydroxy and keto-imide groups in collagen has been suggested in earlier papers [1,2], in which experimental findings supporting this assertion were briefly discussed. The excellent correlation between the content of hydroxyproline and the degree of hydrothermal stability of collagens of a variety of teleostean and mammalian skins [3,4] was one of the principal arguments, further emphasized by the finding that by exhaustive acetylation of bovine collagen (N- and O-acetylation), its shrinkage temperature was lowered by 25° C [1]. Moreover, the changed reactivity of the bovine collagen caused by the inactivation of the hydroxy groups of collagen was found to conform with the postulated crosslinkage [2]. In the present paper, some additional data considered by the author to be indirect proof for the existence of such a stabilizing bond are presented.

EXPERIMENTAL

Materials. a) Substrates. Mammalian collagen in the form of hide powder (Lyon) and modified specimens of this hide powder were employed as substrates. Hide powder collagen of various degrees of acetylation formed the most important substrate of the modified collagens. In the N-acetylation and the exhaustive acetylation (N- and O-acetylation) of the collagen, the technique of Green *et al.*[5] was used. A sample of an intermediate degree of acetylation (complete N-acetylation and about 40 % of the

hydroxy groups acetylated) was included in the series. The treatment was identical with that of the exhaustive acetylation, except that the time of acetylation was reduced from 12 days to 6 days. Further, collagen (hide powder) with its carboxyl groups esterified exhaustively, prepared by the methylation technique of Fraenkel-Conrat and Olcott [6], described earlier [7], was included in the series. Finally, hide powder which had been heat-denatured in water of 70° C for one minute [8], and as a representative of the lyotropic salt effect [9], hide powder pretreated for 4 days in 2 M solution of sodium perchlorate of pH 5.5 at 20° C, subsequently washed and freed from the salt, were employed. The analytical characteristics of these specimens of collagen are given in Table 1.

Table 1. Some analytical characteristics of the specimens of collagens (on 1 g ash- and fat-free dry substance).

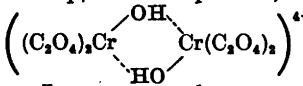
Substrate	% Total N	meq. N-acetyl	meq. O-acetyl	Acid binding capacity in meq. HCl	*T*s in °C
Hide powder (Blank)	18.05	0.00	0.00	0.92	65
N-acetylated hide powder	17.9	0.39	0.00	0.76	66
Partially O-acetylated hide powder	17.2	0.39	1.01	0.72	44
Exhaustively O-acetylated hide powder	16.6	0.39	1.25	0.60	40
Esterified hide powder *	17.3	0.00	0.00	0.11	35
Heat-denatured hide powder (shrunk)	18.0	0.00	0.00	0.92	—

* 2.83 % methoxyl on the protein basis (corrected value).

It should be noted that sodium perchlorate in 2—4 M solutions has proved to be the most excellent lyotropic agent among the numerous neutral salts of this type tested. Its main action on collagen is directed on the non-ionic crosslinks (hydrogen bonds) which are ruptured. In the present instance, the loss of collagen incurred in the brief treatment amounted to 22 % of the original amount. Finally, a modified polyamide [10-12], based on the copolymerisate of adipic acid-hexamethylene salt (60 %) and caprolactam (40 %), containing 12.1 % N, was selected since it supplies a substrate with its reactivity restricted to the coordination faculty of the keto-imide group, the most frequently occurring protein linkage. This special polyamide was made into woolly fibres by stirring the hot saturated methanol solution of the polyamide into water. Its base binding capacity (at equilibrium pH of 12) was only 0.02 meq. per g polyamide [10-12].

b) Interacting agents (Tanning agents). In order not to obscure the main points of the issue involved, only a few examples selected from the numerous tanning agents and complex salts investigated will be given. The reaction of some aromatic compounds with a selective affinity for the hydroxy group will be separately treated in another connection. The main reagents employed were chromium salts, representing various types of reactivity. The typical cationic chromium complexes, the sulphato-oxo-chromium cations, with a composition corresponding to the empirical formula $(Cr_3(OH)_2SO_4)^{3+}$ were supplied by an equilibrated solution of the 67 % acid chromium sulphate $(Cr_3(OH)_2(SO_4)_3) \cdot Na_2SO_4$, at a concentration of 0.4 eq./l. It consisted of 98 % of cationic chromium complexes [13] and is a compound specifically restricted to the carboxyl ions of collagen for its irreversible fixation. A solution of 67 % acid chromium chloride, corresponding to the empirical formula $Cr_3(OH)_2Cl_4 \cdot 2NaCl$ (Ref.[13]) equilibrated at 1.0 eq./l Cr, which contained mainly cationic chromium complexes, represents an agent predominantly fixed by the carboxyl ions of collagen but this reaction, however, is supplemented at the concentration used, by secondary reactions involving the non-ionic protein groups. An extremely basic chromium chloride, corresponding to the formula $Cr_3(OH)_4Cl_2 \cdot 2NaCl$ (Ref.[13]) employed in a solution containing 0.6 eq./l Cr, and composed of 70 % non-ionic chromium complexes and 30 % cationic ones, was included, and also the corresponding chromium perchlorate, at the same chromium concentration, containing 68 % non-ionic chromium complexes and 32 % cationic ones. In the reactions of these solutions with collagen, the non-ionic complexes take a prominent part. Another type of non-ionic chromium complexes is present in the solutions of the sulphite-complexed basic chromium sulphates,

which were prepared by adding 2 moles, and 2.5 moles of sodium sulphite per mole Cr_2O_3, to a salt-free 67 % acid chromium sulphate solution, applied at a concentration of 0.8 eq./l Cr. The complex composition of the solution corresponding to the stoichiometric ratio: $Cr_2(OH)_2(SO_4)_2 \cdot 2Na_2SO_3$, was 80 % non-ionic chromium, 12 % cationic and 8 % anionic complexes [7]. The corresponding figures for the more highly sulphite-masked solution was 86, 0, and 14. The pH values were 4.2 and 4.8, respectively. A similar solution of complexed basic chromium sulphate, in which sodium phthalate was the effective masking agent, employed 1 mole phthalate per mole Cr_2O_3 at 0.8 eq./l Cr and contained highly aggregated chromium complexes, 65 % in the non-ionic form and 35 % in the form of cationic complexes [14]. Finally, a solution of sodium tetraoxalatodiol-chromiate [15,16], 0.8 eq./l Cr and pH 5.6, containing the anionic chromium complex:



was selected as a typical anionic agent.

In some complementary experiments the tanning with a solution of mimosa extract (the condensed type of vegetable tannin), 5 w/v % tannins, pH 4.8, was studied.

Methods. Portions equal to 1.0 g protein, completely hydrated, were shaken with 40 ml of the solutions of the tanning agents for 48 h after which time the equilibrium of the system had been attained in the majority of the series. For some series, notably the oxalato-chromiate, two weeks' reaction time was required, and one week for the mimosa tannage. After complete reaction, the substrates were freed from soluble matter, the point being indicated by the conventional analytical tests on the exhaust water. The analytical determinations were carried out by conventional methods.

RESULTS

It has been established previously [12], that the vegetable tannins of the condensed type, such as the mimosa tannins are predominantly fixed by the keto-imide link of collagen; their binding by polyamides, with the —CONH-group as the sole reacting group, being the crucial evidence [12]. It has also been shown that by heat denaturation of collagen (thermal shrinkage [8]), or by pretreatment of collagen with lyotropic agents in concentrated solution, subsequently restoring the protein to its isoelectric state, the irreversible binding of vegetable tannins is greatly increased [9]. By these modifications of collagen, additional coordination sites (hydrogen bonding) on the non-ionic protein groups, internally compensated in the intact collagen, are made available to the tannins by the rupture of crosslinks, in which such groups are involved.[17]

Table 2 contains a resumé of data on the fixation of mimosa tannins by the various substrates.

Table 2. The fixation of Mimosa tannins by various substrates (in % tannin fixed by the protein).

No.	1	2	3	4	5	6	7	8
Type	*Esterified hide powder*	*Polyamide*	*Hide powder (=h.p.)*	*Shrunk h.p.*	*H.p. pretreted in 2 M $NaClO_4$*	*N-acetyl. h.p.*	*Partially O-acetyl. h.p.*	*Exhaustively acetylated h.p.*
% fixation	124	97	60	96	118	56	78	93

In Table 3 the data on the fixation of the chromium compounds by the various substrates are summarized.

Table 3. The fixation of chromium complexes by the various substrates (in % Cr_2O_3 fixed by the protein).

No.	1	2	3	4	5	6	7	8
Solution	*Polyamide*	*Esterified collagen*	*Hide powder (=h.p.)*	*Shrunk h.p.*	*H.p. pretreted in 2 M $NaClO_4$ solution*	*N-acetyl h.p.*	*Partially O-acetylated h.p.*	*Exhaustively acetylated h.p.*
67 % acid Cr-sulphate	0.2	0.1	10.2	11.0	11.9	8.2	7.8	7.0
67 % acid Cr-chloride	0.3	0.4	6.6	8.8	6.2	6.5	7.6	7.8
33 % acid Cr-chloride	2.5	3.1	12.4	18.1	21.3	11.2	15.5	18.7
33 % acid Cr-perchlorate	7.2	2.6	8.4	14.1	15.9	7.7	10.0	12.4
Sulphito-Cr-sulphate ($2SO_4$:$1Cr_2O_3$)	0.2	21.0	23.9	37.0	40.7	19.2	—	20.8
Sulphito-Cr-sulphate ($2.5SO_3$:$1Cr_2O_3$)	0.9	40.6	29.4	53.8	60.3	25.8	24.1	22.7
Phthalato-Cr-sulphate	4.5	7.0	17.2	27.5	30.4	15.2	—	15.8
Oxalato-chromiate	0.1	13.6	6.7	12.4	13.9	5.9	—	5.1

DISCUSSION

The data on the fixation of the vegetable tannins by the various collagen preparations indicate that by the pretreatments in which rupture of crosslinks of the hydrogen bond type has been proved to occur, such as in the series Nos. 1, 4, 5, and 8, the combining power of the collagen for these coordination agents is increased by about 50 % of the values obtained from the intact collagen. It should be noted that exhaustive acetylation, *i. e.*, acetylation involving the hydroxy group, is as effective in augmenting the fixation of the polyphenolic agent as is the straight breaking of crosslinks in the collagen lattice by supplying energy to the system. Also the methylation of the carboxyl groups of collagen (No. 1) which is carried out at a pH value as low as 1 in methanol, results in the breaking of bonds in which groups forming binding sites for the vegetable tannins are freed, and additional binding sites made available to the tannins. As already mentioned, the very marked tannin uptake by the polyamide, *i. e.*, by means of the —CO—NH-link, supports the concept of the increased tannin fixation resulting from the pretreatments mentioned being mainly due to the free sites of reaction on the keto-imide groups resulting from the dislocation of hydrogen bonds in which the peptide link partakes. Obviously, these systems supply no information whether the additional coordination sites on the —CO—NH-groups are due to the breaking of hydrogen bonds between two adjacent keto-imide linkages, or to the rupture of the hydroxy-keto-imide link, or to both reactions. These findings of the changed reactivity of the modified collagens towards an agent which is attached chiefly to the keto-imide group are important for the elucidation of the reaction of non-ionic chromium complexes with collagen.

For a discussion of the data in Table 3, and particularly to enable conclusions to be drawn from them, it is advisable didactically to discuss the data obtained with solution No. 1 first, *i. e.*, the cationic chromium complexes which do not react with substrates lacking in carboxyl ions, such as the polyamide and the esterified collagen [11]. By heat denaturation, the ionic groups of collagen are not made more reactive or increased [8]. Hence, the cationic chrome fixation should not be affected by the hydrothermal shrinkage of collagen, as is the case [8]. The severe lyotropic pretreatment (No. 5), inducing very great swelling of the collagen, evidently results in secondary breaking of cross-links in which the carboxyl group is involved, the result being slightly increased uptake of the cationic chromium complexes.

By inactivation of the amino group by acetylation (No. 6), the chrome fixation is lowered. This decrease is of the order generally found for collagen in which the amino group has been removed or inactivated [18]. This impairment has been shown to be a secondary effect of the decreased uptake of the protolyzed acid of the system, which change retracts on the chrome fixation that is intimately bound up with the removal of protolyzed acid by collagen [18]. The same trend, but still more pronounced is shown by the O-acetylated specimens which indicates that the hydroxy group is apparently involved in the creaction.

The more concentrated solution of the 67 % acid chromium chloride which contained preponderantly [13] cationic chromium complexes, was intentionally selected, since it presents a more complicated type of reaction. Apart from the primary ionic fixation, the main reaction, the occurrence of another mode of reaction is indicated at the chromium concentration employed. It should be noted that from dilute solutions of this chromium compound the chrome fixation is entirely ionic, located solely on the carboxyl ions of the collagen. Thus, the increased reactivity of the non-ionic protein groups resulting from heat denaturation or the lyotropic salt effect augments the chromium uptake from the more concentrated solution by about 25 % of the amount fixed by the intact collagen. The N-acetylation has no effect on the reaction, but after O-acetylation of collagen an increased chromium fixation is noted. Apparently, come groups are freed by the exhaustive acetylation which fix the non-ionic somplexes present in the solution of the basic chloride.

A more favourable case for the interpretation of the reaction mechanism is the extremely basic chromium chloride (33 % acidity) solution, in which non-ionic chromium complexes predominate [13]. It possesses only a slight affinity for the keto-imide groups (the polyamide). Hence, the drastically increased chrome fixation, effected by the thermal shrinkage and particularly by the lyotropic pretreatment, must be due to the freeing of some other group than the keto-imide linkage in these denaturations. In view of the inert behaviour of these chromium complexes to the polyamide, it is difficult to explain the marked effect of the O-acetylation *i. e.*, the inactivation of the hydroxy groups, on the uptake of chromium. It is possible that the presence of some other protein group is required for its fixation by means of the —CO—NH-link which is absent in the polyamide. At the present state of our knowledge, the reaction cannot be explained.

The corresponding perchlorate (33 % acid) shows a great affinity for the polyamide, the amount of chromium fixed in this instance being about 85 % of the amount fixed by collagen. This marked increase of the binding of chromium (from 8.4 to 12.4 % Cr_2O_3), resulting from the inactivation of the hydroxy groups, is satisfactorily accounted for by the additional reaction sites on the —CO—NH-links formed by the breaking of the —OH----OC-NH- bonds in the O-acetylation.

The data from the series with solutions of the non-ionic sulphitochromium sulphates supply the *crucial* experimental support for the view postulating the presence of a stable link: the hydroxy group being internally compensated by a strong hydrogen bond to the oxygen atom of the keto-imide group. The polyamide has *no* affinity at all for the sulphito complexes [11], while the esterified collagen binds greater amounts of these complexes than the intact collagen [7], accounted for by the extensive rupture of coordinate crosslinks (hydrogen bonds) in the heavy swelling taking place in the methylation process, as mentioned already (T_s lowering of 30° C). By the thermal shrinkage and the lyotropic pretreatment, the fixation of the non-ionic sulphito complexes by collagen is tremendously increased [8,9]. The most convincing proof of the participation of the hydroxy group in this reaction concerns the effect of the O-acetylation which results in a very marked decrease of the irreversible fixation of chromium by collagen. The only explanation which conforms with all the facts cited and with other findings which cannot be enumerated in this connection is that the —OH----OC-bond in collagen is broken by the acetylation of the hydroxy group and thus the compensation on the —CO—NH-group is removed and it is made coordinatively reactive. This will result in the augmentation of reactions on the keto-imide group (by hydrogen bonding), as for instance the uptake of polyphenolic vegetable tannins and the non-ionic chromium perchlorates, while the fixation of the non-ionic sulphito-chromium complexes, which rely on the hydroxy groups for their attachment to collagen, is impaired.

It is to be noted that the pretreatments of collagen which would be expected to rupture the postulated crosslink, setting free both the OH group and the —CO—NH-group have a very favourable effect on the binding of the sulphito complexes, and indicate that they are bonded to the freed OH-groups.

It was deemed of interest to include in these series compounds with a tanning potency expected to be similar to that of the sulphito-chromium sulphate. The solution of the phthalato-chromium sulphate, obtained by the addition of one mole of sodium phthalate to each mole Cr_2O_3 of the 67 % acid salt-free chromium sulphate solution was used. The phthalate is a powerful complexing agent for chromium [19], and also markedly increases the size of the masked chromium complexes by bridging the chromium atoms [19]. By the extent of masking employed, about two thirds of the cationic sulphato-oxo-chromium cations were converted into non-ionic phthalato-chromium complexes [14]. As to the reactivity of these complexes, it should be noted that they have a marked affinity for the polyamide, about two thirds of the amount of chromium taken up by the esterified collagen being fixed. This fact is of importance from the point of view of the non-reactivity of the non-ionic sulphito complexes with the polyamide, since their inertness cannot be due to

their large molecular size, hindering diffusion into the polyamide, in view of the very large fixation of the still more highly aggregated phthalato-chromium complexes, and also of vegetable tannins with molecular weight of one to two thousand. The uptake of the phthalato complexes by collagen is slightly impaired by its N-acetylation, which is probably due to the indirect effect of the blocking of the amino groups on the cationic chromium fixation as already mentioned. By exhaustive acetylation (including the OH group), no further lowering of the chromium uptake than that noted for the N-acetylated specimen is found. Since by the O-acetylation, the hydroxy group is inactivated, while the keto-imide group is activated coordinatively, the independence of the chromium fixation noted in this instance might be interpreted as implying that both the protein groups which form this hydrogen bond, function as binding sites for the phthalato-chromium compounds. The increased reactivity to be expected from the freeing of the —CO—NH-link from its compensation by the hydroxy group, is then balanced by the blocking of the potential coordination sites on the OH groups. For the non-ionic chromium compounds specifically directed on to the keto-imide group, the O-acetylation results in a heavy increase of the chrome fixation, as earlier noted. The final result of the moderate lowering of the amount of chromium fixed, compared to intact collagen, effected by the inactivation of the hydroxy groups and the activation of the keto-imide group, should thus exclude a reaction exclusively governed by the keto-imide-coordination but favour the occurence of two reactions of the opposite trend, almost counteracting each other.

Finally, the combination of the tetraoxalato-diol-chromiate with the various substrates represents the reactions of an anionic complex. This has no affinity for the polyamide, excluding the participation of the polypeptide link in its attachment to collagen. By the esterification of collagen the chrome fixation is more than doubled. Besides making a greater number of cationic protein groups available for the chromiate by the discharge and inactivation of the carboxyl ions in the esterification, the freeing of non-ionic protein groups by dislocation of various hydrogen bonds in the collagen structure (swelling) is probably responsible for the increased reactivity noted, further supported by the data on the effect of the denaturation processes. By exhaustive acetylation the chrome fixation is impaired to a greater extent than by N-acetylation, indicating that the hydroxy groups are taking some part in the binding of this type of chromium complexes. The marked influence of the heat denaturation of collagen and the lyotropic effects on the binding of the oxalato complexes, practically doubling the fixation, should be viewed in the light of the inability of the —CO—NH-groups of the polyamide to react with these complexes. Moreover, considering that the reactivity of the cationic protein groups is not altered by these pretreatments of collagen [9], the conclusion seems justified that the hydroxy groups form sites for the binding of a part of the oxalato-chromiate, particularly in the later stage of reaction. For the participation of the cationic protein groups, adequate experimental proofs have been adduced earlier [15,16]. It is of interest to note that the reaction of the oxalato compounds with collagen occurs at a very slow rate [16], several weeks of tanning being required for attainment of equilibrium, while the corresponding point in the tanning with cationic complexes, *i. e.*, reactions restricted to

the free carboxyl ions of collagen, is already reached after only a few days [20]. The participation of hydroxy groups gradually freed from their compensation and made available to the oxalato complexes would satisfactorily explain the time factor. A similar trend of the rate of fixation is shown by the sulphitochromium compounds. Further, some type of coordination of the hydrogen bond type is probably involved in the reaction since a considerable part (about a third in the present instance) of the oxalato complexes fixed by collagen are removed on treatment with 6—8 *M* solutions of urea, which specifically removes hydrogen bonded compounds from their attachment on collagen [21].

Summing up, the different trends of the data on chrome fixation by collagen after its acetylation and denaturation, with regard also to the behaviour of the chromium compounds studied with the polyamide (coordination on the —CO—NH-group), appear to be adequately explained by the assumption of the presence of a cross-link of the type —OH----OC$\begin{matrix}\diagup\,\text{NH}— \\ \diagdown\end{matrix}$ in collagen, which is severed by certain pretreatments of collagen. It is obvious that the postulated link, which is supported by numerous other findings [1,2], does not detract from the importance of the hydrogen bridges between adjacent ketoimide groups, generally conceived to be the principal stabilizing crosslinks in proteins.

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Surface-Chemical Studies on the Formation of Aluminium Soaps

III. The Interaction between Aluminium Ions and Myristic Acid Monolayers [1,2]

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The conditions promoting interaction between the aluminium ions of potassium aluminium sulphate solutions and myristic acid monolayers spread on these solutions have been studied with a continuously recording surface balance. The conditions in which the interaction begins, in which it attains a wider scope and in which it leads to a definite endpoint are well-defined and reproducible. The monolayer properties of the aluminium myristate formed by the interaction have been examined. Data characteristic of the monolayers are given.

For our study of the conditions favouring the formation of aluminium soaps from fatty acids, we have chosen myristic acid to represent the latter, particularly as the monolayer properties of myristic acid at various substrate pH values have been previously investigated in detail in this laboratory [3]. In view of the fact that myristic acid forms monolayers of the expanded type at room temperature, it was to be expected that the interaction with aluminium ions would from the beginning be revealed by the condensing effect of these ions upon the monolayers.

The melting point of the myristic acid employed in the study was 54.04° C. The experimental method and the apparatus were the same as those employed previously[1].

MYRISTIC ACID MONOLAYERS AT VARIOUS pH VALUES

The surface pressure-area curves of myristic acid monolayers are well known. A curve recorded on dilute hydrochloric acid (pH 2.5) is reproduced in Fig. 1. The notation used to designate various characteristic points on this pressure-area curve will be employed also in the following.

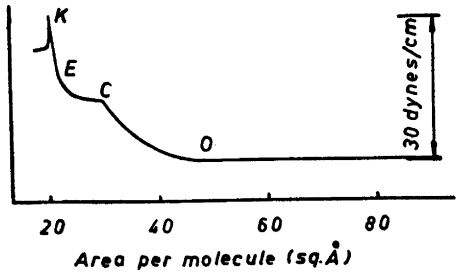

Fig. 1. π—A curve of myristic acid recorded on dilute hydrochloric acid (pH 2.5). 20° C.

Figs. 2a and 2b show how the area and pressure values change when the pH of the substrate is varied (by adding hydrochloric acid and sodium hydroxide; no buffer salts were used in this study).

When the substrate contains no added sodium chloride, most of the area and pressure values remain practically constant up to a pH value of 5.4 (Fig. 2a); only the collapse pressure, π_K, undergoes a slight increase above pH 3.5. On a substrate containing 3 moles of sodium chloride per litre (Fig. 2b), the values remain unaltered up to pH 3.5, except π_K, which begins to increase as soon as the pH rises to 2.5. The changes that occur in the myristic acid monolayers above the pH values mentioned are doubtless connected with the

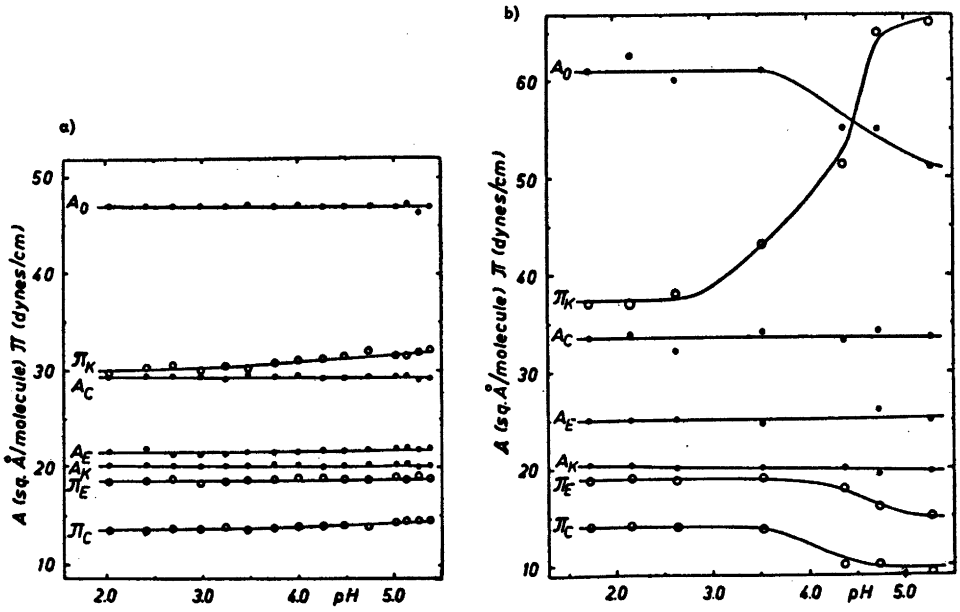

Fig. 2. The dependence of some characteristic points on the pressure-area curves on the pH of the substrate (pH adjusted with dilute HCl or NaOH). 20° C. a. The substrate contains no added sodium chloride. b. The substrate contains 3 moles of sodium chloride per litre.

beginning ionization of myristic acid; these will be discussed elsewhere [3-6]. In Table 1 data are given which characterize the properties of monolayers of undissociated myristic acid under different conditions.

Table 1. Characteristic data for undissociated myristic acid monolayers. The pH of the substrate has been varied by adding HCl or NaOH.

Substrate	Areas per molecule, sq. Å				Surface pressures, dynes/cm		
	A_O	A_C	A_E	A_K	π_C	π_E	π_K
No NaCl added (pH<3.5)	47.0	29.4	21.6	20.2	13.6	18.6	30.4
3 M NaCl (pH<2.5)	63.0	33.5	24.8	20.3	14.0	19.0	37.5

INTERACTION BETWEEN ALUMINIUM IONS AND MYRISTIC ACID MONOLAYERS

a) Substrates containing no added sodium chloride.

The pressure-area curve recordings were made in some cases at constant pH values and with different aluminium ion concentrations, in other cases with constant aluminium ion concentrations and various pH values.

In Fig. 3 are shown a series of pressure-area curves recorded on substrates of pH 3.62 containing various concentrations of potassium aluminium sulphate. At this pH value the monolayer is on aluminium-free substrates almost solely composed of undissociated myristic acid. It is evident from the figure that the expanded pressure-area curve typical of undissociated myristic acid is gradually transformed into a curve of the condensed type as the aluminium ion concentration increases. This transformation is accompanied by a gradual disappearance of the liquid expanded (O—C) and the intermediate (C—E) sections of the pressure-area curve and by a change in the form of the upper part of the curve. This latter change has already occurred in curves 6 and 7, but the middle region of the curve attains its final form (curves 9 and 10) only after the aluminium ion concentration has been increased to 1.6×10^{-3} M. The new curve type does not undergo any further change when the aluminium ion concentration is increased to forty times this value. It is obvious that the changes must be ascribed to the gradual transformation of the monolayer from one containing only myristic acid to one composed of some kind of aluminium myristate, which transformation is complete only after the curve of the new form is fully developed.

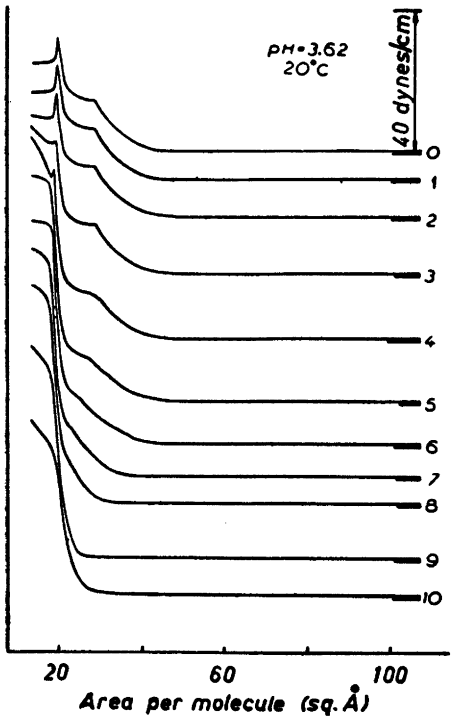

Fig. 3. π—A curves of myristic acid recorded on substrates containing different potassium aluminium sulphate concentrations. pH 3.62. 20° C.

Curve No.	*Al concentration moles/litre*
0	—
1	$3.0 \cdot 10^{-6}$
2	$1.0 \cdot 10^{-5}$
3	$5.0 \cdot 10^{-5}$
4	$2.0 \cdot 10^{-4}$
5	$4.0 \cdot 10^{-4}$
6	$7.0 \cdot 10^{-4}$
7	$1.0 \cdot 10^{-3}$
8	$1.2 \cdot 10^{-3}$
9	$1.6 \cdot 10^{-3}$
10	$1.3 \cdot 10^{-2}$

The variation of the monolayer properties with increasing aluminium ion concentration in the substrate is shown in Fig. 4. In this figure the values for the various characteristic points on the curves are plotted against the negative logarithm of the aluminium ion concentration.

At the pH value in question, 3.62, the myristic acid monolayer is not affected by the aluminium ions before the concentration of the latter has exceeded *ca.* 5×10^{-6} M. At this point the collapse pressure, π_K, begins to increase. At higher concentrations it passes through a maximum and finally decreases. At still higher aluminium ion concentrations (about 1.5×10^{-2} M), the collapse pressure again begins to rise.

The values corresponding to the other characteristic points in the curves deviate from those typical of the pure myristic acid monolayer only at appreciably higher aluminium concentrations, at about 5×10^{-5} M. The area per molecule at point 0, A_0, decreases gradually to two-fifths of its initial value, and then begins to increase again at higher aluminium ion concentrations. The point C divides into two separate break points C_I and C_{II}, the former shifting to lower and the latter to higher area values. The surface pressures relating to these points decrease in value, that for the former slowly and that for the latter more rapidly. These break points disappear from the curve completely when the aluminium content of the substrate is increased above 10^{-3} M. Above this

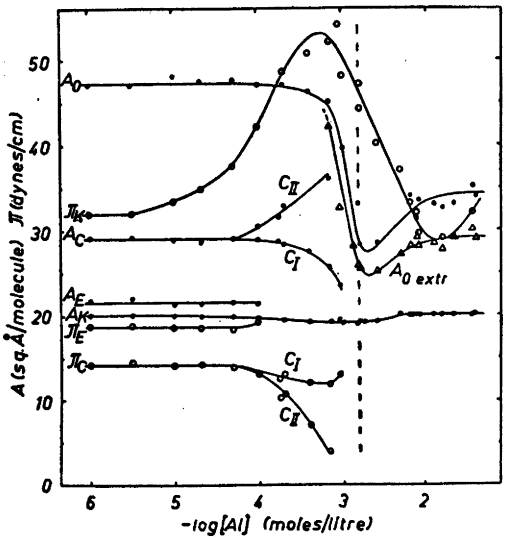

Fig. 4. The dependence of some characteristic points on the pressure-area curves on the potassium aluminium sulphate concentration of the substrate at constant pH 3.62. 20° C.

latter concentration the curve may be considered to comprise only two main parts joined by a short curved section, one which runs almost parallel to the horizontal axis and another which rises abruptly. The value of the extrapolated point of intersection, $A_{0\ extr}$, changes parallel with A_0 . The collapse area, A_K, varies only slightly, decreases slightly at first but later reverts to its initial value with increasing aluminium concentration.

The monolayer thus undergoes very little change when the aluminium ion concentration is raised to 5×10^{-5} M. Above this latter point there follows an aluminium ion concentration range between 5×10^{-5} and 1.6—2.5×10^{-3} M where the monolayer properties undergo marked variations, but then there follows a range where the monolayer properties vary rather little with the aluminium concentration. In the intermediate concentration range (5×10^{-5}—2×10^{-3} M) the transition to the new curve type occurs; this type is fully developed at the upper limit of the concentration range.

Several series of experiments were also conducted in which the aluminium ion concentration of the substrate was held constant, but the pH was varied. The data from these series are plotted in Figs. 5—9.

The figures give values for the characteristic points of the pressure-area curves at different pH values up to those where aluminium hydroxide begins to precipitate, *i. e.*, up to the pH values 4.0—5.4 (depending on the aluminium ion concentration). Up to these pH values the properties of a monolayer of pure myristic acid undergo very little change as we have seen above. When aluminium ions are present in constant concentration in the substrate, however, the values for the characteristic points undergo marked variation with

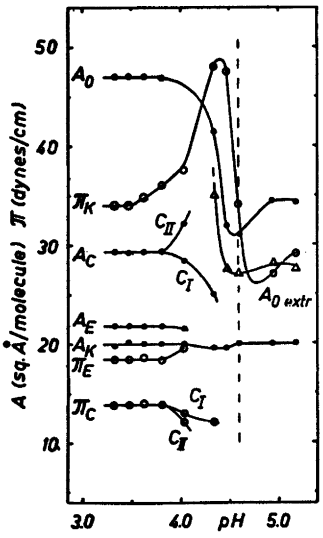

Fig. 5. 6.3 · 10⁻⁶ mole Al per litre

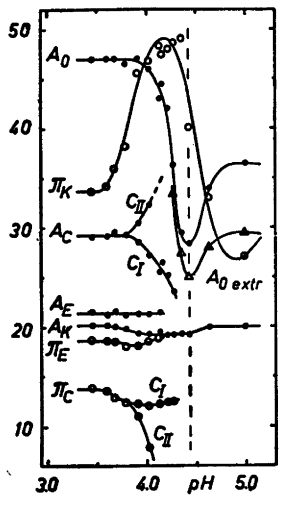

Fig. 6. 1.0 · 10⁻⁵ mole Al per litre

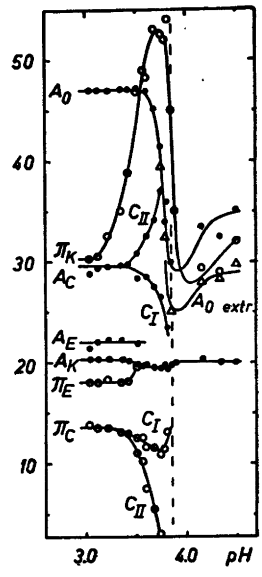

Fig. 7. 2.5 · 10⁻⁴ mole Al per litre

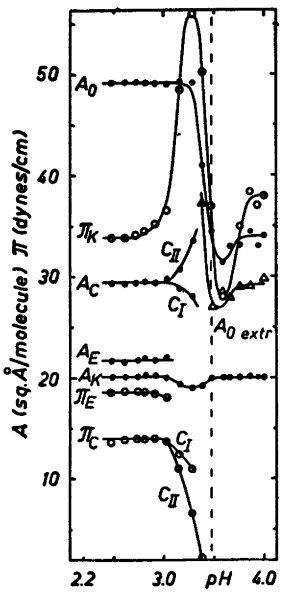

Fig. 8. 1.0 · 10⁻³ mole Al per litre

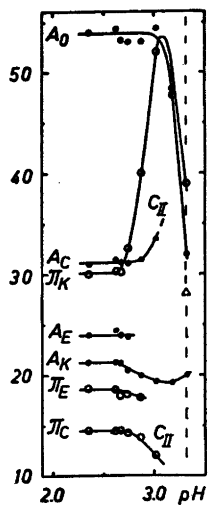

Fig. 9. 6.0 · 10⁻³ mole Al per litre

Fig. 5—9. The dependence of some characteristic points on the pressure-area curves on the pH of aqueous potassium aluminium sulphate solutions.

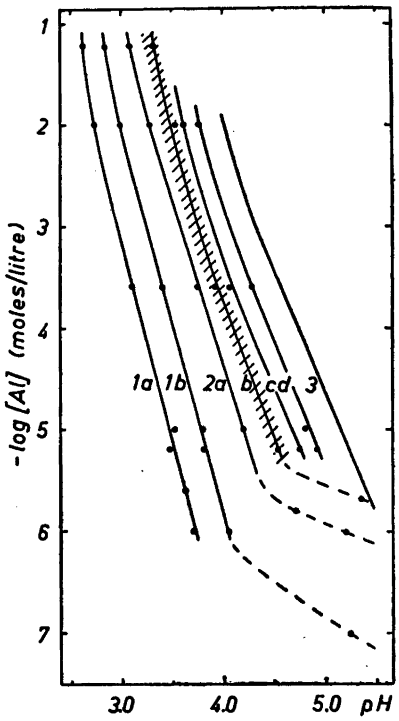

Fig. 10. Diagram showing the conditions in which interaction occurs between myristic acid monolayers and aluminium ions in aqueous solutions containing $KAl(SO_4)_2$. Curves 1a and 1b give the aluminium concentrations and pH values of the substrate when the first signs of interaction between aluminium ions and myristic acid are noted. Curves 2a—2d connect the aluminium concentrations and pH values at which maximum interaction is observed. Curve 3 gives the aluminium concentrations and pH values at which aluminium hydroxide precipitates in equilibrium conditions. 20° C.

pH, similarly as when the aluminium ion concentration was gradually increased. This shows that the myristic acid monolayer is gradually transformed into an aluminium myristate monolayer when the pH of the substrate is increased. The type of curve characteristic of monolayers of the latter substance is fully developed at the pH values indicated by a dashed line in the figures.

Also in these cases it is the collapse pressure, π_K, that first begins to change; it passes through a pronounced maximum and then a minimum. The other quantities begin to change at pH values that are about 0.2—0.4 unit higher; the greatest variation is shown by the area per molecule at the point 0, A_0, which decreases rapidly to a deep minimum. It is seen that the higher the aluminium concentration, the lower the pH values where the changes occur.

The results of the above experiments on the interaction between myristic acid monolayers and aluminium ions in the substrate are summarized in Fig. 10. The curves 1a and 1b give the conditions at which the first signs of interaction are observed (1a: π_K begins to increase; 1b: A_0 begins to decrease, A_C, π_C and A_K begin to change). The curves 2a, 2b, 2c, and 2d give those conditions at which the maximum changes are noted, *i. e.*, the conditions at which the various quantities attain maximum or minimum values (2a: π_K passes through a maximum; 2b: A_0 decreases to a minimum value; 2c: π_K attains a minimum value; 2d: A_0 increases to an almost constant value). Curve 3 gives those equilibrium conditions at which precipitation of aluminium hydroxide

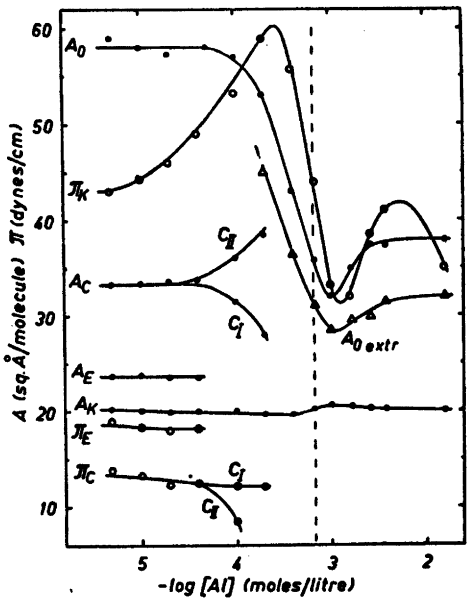

Fig. 11. The dependence of some characteristic points on the pressure-area curves on the potassium aluminium sulphate concentration of the substrate at constant pH 3.60. 20° C. The substrate contained 3 moles of sodium chloride per litre.

begins. The shaded region gives the lowest aluminium ion concentrations and pH values where the fully developed aluminium myristate curve type is observed.

At pH values and aluminium ion concentrations that lie to the left of the curves 1, no interaction has been detected between the aluminium ions of the substrate and the myristic acid in the monolayer. In the region between curves 1 and 2 interaction is reflected in the variation of the different quantities, the greatest interaction taking place within the region between curves 1b and 2b. In the vicinity of the latter curve the monolayer of myristic acid seems to have been completely transformed into an aluminium myristate monolayer.

The full-drawn curves give the conditions in which the interaction is independent of the time that has elapsed before the compression of the monolayer is begun. At low aluminium concentrations the interaction requires some time to attain equilibrium; the dotted parts of the curves 1 and 2 apply to the time of reaction allowed in our experiments, *i. e.*, a total reaction period of five minutes. If the aluminium ions are given a longer time to diffuse to the myristic acid monolayer, the first signs of interaction will be observed at somewhat lower aluminium ion concentrations and lower pH values.

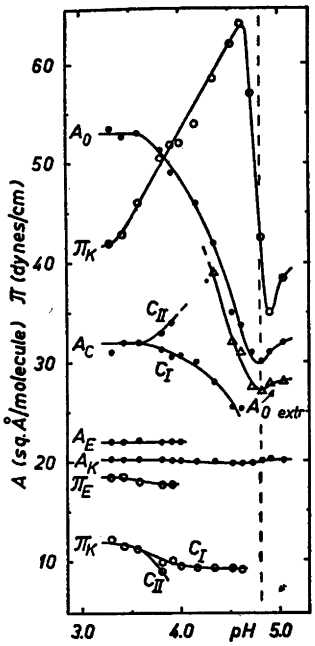

Fig. 12. 1.0 · 10⁻⁵ mole Al per litre.

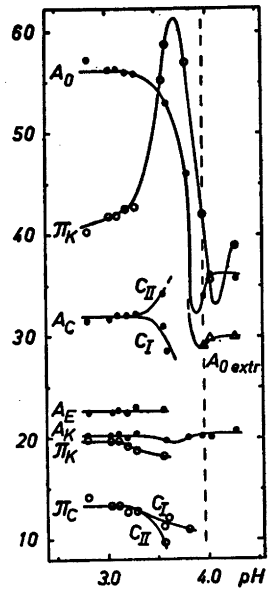

Fig. 13. 2.0 · 10⁻⁴ mole Al per litre.

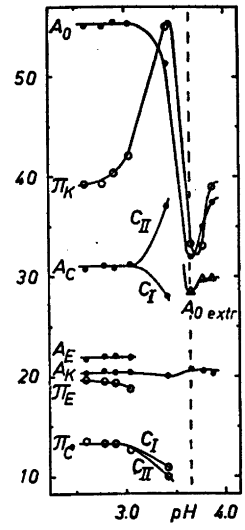

Fig. 14. 1.0 · 10⁻³ mole Al per litre.

b) Substrates containing 3 moles of sodium chloride per litre

In order to eliminate the possible effects of the slight solubility of the monolayer substance in the substrate, a series of experiments was conducted employing a substrate containing a relatively high concentration of sodium chloride. The effect of aluminium ions was found to become evident in a similar manner as in the preceding experiments. Fig. 11 shows data obtained in a series of experiments in which the substrate pH was held constant at 3.60 and the aluminium ion concentration was varied. The data given in Figs. 12—16 were obtained in five experimental series in which the aluminium sulphate content of the substrate was constant (at five levels) and the pH was varied. The notation is the same as that used above.

A summary of the experimental data is shown in Fig. 17 which has been drawn according the same principles as Fig. 10. In the range of conditions to the left of the curves 1, no interaction between the myristic acid monolayer and the aluminium ions was detected, while in the region between curves 1 and 2, the interaction was extensive. The greatest changes in the monolayer were observed in the region between the curves 1b and 2b. In the vicinity of the latter curve, the pressure-area curve type characteristic of the aluminium

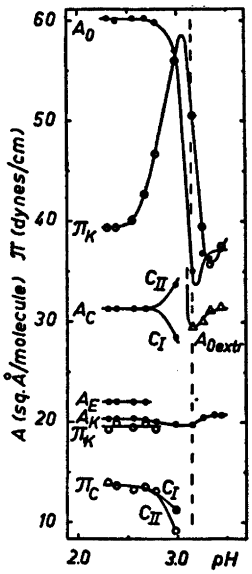

Fig. 15. $6.3 \cdot 10^{-3}$ mole Al per litre.

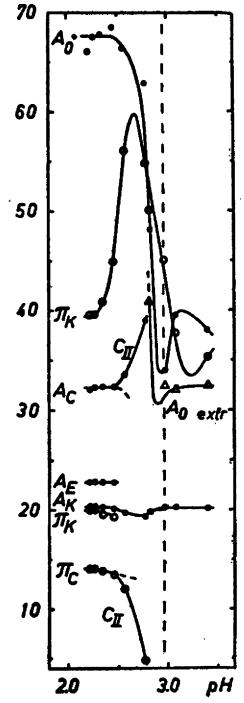

Fig. 16. $4.0 \cdot 10^{-2}$ mole Al per litre.

Fig. 12—16. The dependence of some characteristic points on the pressure-area curves on the pH of aqueous $KAl(SO_4)_2$ solutions containing 3 moles of sodium chloride per litre.

myristate monolayer is fully developed (shaded region), which suggests that the myristic acid has been completely transformed into an aluminium salt.

c) The properties of aluminium myristate monolayers

When interaction takes place between a rosin acid monolayer and aluminium ions of the substrate, all the monolayer properties undergo change almost simultaneously, *i. e.*, when the aluminium ion concentration and the pH attain certain definite values. The same is not the case, however, when a myristic acid monolayer is involved. The various properties characteristic of the monolayer begin to change in succession: the maximum of the collapse pressure, the minimum of the A_0 area and the minimum area at collapse are not attained at the same pH and the same aluminium ion concentration. Neither are the conditions of pH and aluminium ion concentration in all cases the same when the collapse pressure decreases to a minimum and the area A_0 attains a

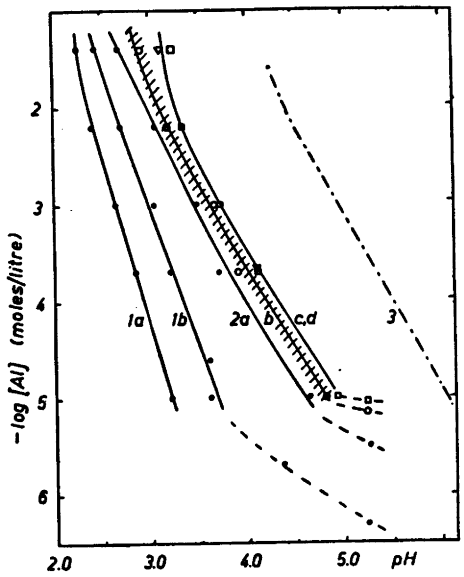

Fig. 17. Diagram showing the conditions in which interaction occurs between myristic acid monolayers and aluminium ions in aqueous solutions of $KAl(SO_4)_2$ containing 3 moles of sodium chloride per litre. Curves 1a and 1b give the aluminium concentrations and pH values of the substrate when the first signs of interaction between aluminium ions and myristic acid are noted. Curves 2a—2d connect the aluminium concentrations and pH values at which maximum interaction is observed. (Curve 3 gives the aluminium concentrations and pH values at which aluminium hydroxide precipitates in equilibrium conditions, when the solution contains 2 moles of sodium chloride and $5 \cdot 10^{-2}$ moles of acetic acid-acetate buffer per litre.)

new fairly constant value at higher aluminium concentrations and pH values. In the case of myristic acid it is therefore more difficult to determine when the reaction goes to completion. This implies either that the reaction between aluminium ions and myristic acid passes through a series of more or less well-defined stages or that the transition of the monolayer properties to those of the final product is complicated by, *e. g.*, steric factors.

As we have seen above, the pressure-area curve type of the aluminium myristate monolayer is in no case fully developed when the collapse pressure, π_K, passes its pronounced maximum. This curve type develops only in those conditions where the molecular area A_0 attains its minimum value. This suggests that the formation of an aluminium myristate monolayer is more clearly indicated by the changes which the properties of a "spontaneously" condensed monolayer, for example A_0 and $A_{0\ extr}$, undergo than by the changes in the properties of the strongly compressed monolayer, *i. e.* π_K and A_K.

We hence believe that it is correct to characterize the aluminium myristate monolayer by its properties under those conditions where A_0 attains its minimum value and where A_0 becomes constant.

Table 2. Characteristic data for aluminium myristate monolayers.

Substrate	A. At minimum A_0				B. When A_0 becomes constant			
	Areas per molecule, sq. Å			Surface pressure dynes/cm	Areas per molecule, sq. Å			Surface pressure, dynes/cm
	A_0	$A_{0\,extr}$	A_K	π_K	A_0	$A_{0\,extr}$	A_K	π_K
Water, no added sodium chloride (Aluminium ion concentration between 6.0×10^{-6}—1.0×10^{-2} M)	28—32	25—27	19—20	28—42	33—36	28—30	20	27—35
3 M sodium chloride (Aluminium ion concentration between 1.0×10^{-5}—4.0×10^{-2} M)	30—34	27—31	20—21	32—48	33—40	28—33	20—21	33—35

The data in Table 2 have been obtained on substrates with very different aluminium ion concentrations. It is seen that an increase in the sodium chloride content of the substrate leads to larger areas per molecule of monolayer substance and also causes the collapse pressure to increase slightly.

Acknowledgment. The work has been carried out in collaboration with and with the financial support of the *O.Y. Keskuslaboratorio-Centrallaboratorium A.B. (The Research Institute of Finnish Pulp and Paper Industries)*, Helsingfors, Finland.

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Surface-Chemical Studies on the Formation of Aluminium Soaps

IV. The Product of the Reaction between Aluminium Ions and Monolayers of Myristic Acid

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Monomolecular layers of myristic acid have been spread on substrates containing potassium aluminium sulphate under conditions in which interaction takes place between aluminium ions and the monolayer. The interacted layers have been skimmed from the surface and the content of aluminium in the collected substances has been determined.

The final product of the interaction seems to be a dibasic aluminium monomyristate. Typical aluminium myristate monolayer properties, however, appear already when the aluminium content of the monolayer substance has increased to one mole of aluminium to three to two moles of myristic acid.

In the foregoing paper (Part III[1]) we reported on the conditions in which reaction takes place between a myristic acid monolayer and aluminium ions in the supporting substrate. The lowest aluminium concentration at which the reaction begins varies with the pH of the substrate; those aluminium concentrations in which the reaction proceeds through certain intermediate stages and those in which it attains a definite end-point also vary with the substrate pH. As a result of the reaction the monolayer changes from an expanded to a condensed type. In the following we shall describe the results of our investigation on the constitution of the monolayer substance.

THE ISOLATION AND ANALYSIS OF THE MONOLAYER SUBSTANCE

The monolayer substance was collected for analysis in the same manner as in the case of aluminium monorosinate [2] by skimming the monolayer of myristic acid from the surface of the substrate containing potassium aluminium sulphate. The conditions in which the collection of monolayer substance was performed and the amounts collected are given in Table 1.

The aluminium content of the isolated monolayer substance was determined by the surface-chemical method described in Part II [2] of this series. The carbon and hydrogen contents were determined by micro combustion analysis *.

In all cases the analysed substances were those formed under the conditions where the characteristic surface pressure-area curve for the aluminium myristate was fully developed. The material for analyses 1—3 (Table 1) was collected under conditions where the area A_0 in the pressure-area curve was near its minimum value, material for analyses 4—6 when π_K attained its minimum value and material for analyses 7—8 when A_0 became constant.

Table 1. Data defining the conditions in which the analysed monolayer substances were formed and the results of the analyses.

Analyses No.	Conditions for the formation of the monolayer substances		Number of skimmings	Quantity of monolayer substance in mg	Results of the analyses		
	Al conc. M	pH			Al %	C %	H %
1	$1 \cdot 10^{-2}$	3.43	320	5.2	3.5	65.4	10.4
2	$4 \cdot 10^{-2}$	3.56	320	4.0	3.6	63.6	10.2
3	$1 \cdot 10^{-3}$	3.72	360	6.5	4.6	—	—
4	$1 \cdot 10^{-3}$	3.95	360	8.0	5.9	—	—
5	$1 \cdot 10^{-3}$	3.96	360	11.5	7.0	62.6	10.3
6	$1 \cdot 10^{-3}$	3.96	360	12.0	7.3	61.3	10.5
7	$1 \cdot 10^{-3}$	4.27	360	13.5	9.7	—	—
8	$1 \cdot 10^{-3}$	4.28	360	13.3	10.6	—	—

The samples of the first group were found to contain from 3.5 to 4.6 % aluminium. These values correspond most closely to the aluminium content of aluminium trimyristate (Table 2). For the samples of the second group, the aluminium contents were found to be between 5.9 and 7.3 %, which agrees most closely with the aluminium content of aluminium dimyristate or that of a mixture of the latter salt and aluminium monomyristate. The aluminium contents for the samples of the third group correspond to that of dibasic aluminium monomyristate (Table 2). The carbon contents indicate samples composed of mixtures of di- and monomyristates, while the corresponding hydrogen contents are in close agreement with the hydrogen content of aluminium monomyristate. We are inclined to consider the aluminium determinations most reliable for drawing conclusions about the nature of the monolayer substance.

* The latter analyses were performed by Mr. K. Salo at the Chemical Institute of the University of Helsingfors.

Table 2. Theoretical aluminium, carbon and hydrogen contents for different aluminium myristates.

Aluminium myristate	Al %	C %	H %
Dibasic aluminium monomyristate 	9.36	58.3	10.3
Monobasic aluminium dimyristate 	5.42	67.3	11.1
Aluminium trimyristate 	3.81	71.2	11.5

THE RELATIONSHIPS BETWEEN THE ALUMINIUM CONTENT AND PROPERTIES OF THE MONOLAYER SUBSTANCE

Despite the uncertainity of the analytical data, these can be taken to indicate that the aluminium content of the monolayer substance increases to one mole of aluminium to three to two moles of myristic acid simultaneously as the pressure-area curve becomes transformed into the form we have considered typical of the aluminium myristate monolayer. Only under the conditions represented by curve 2d in Fig. 10 of Part III of this series does the aluminium content of the monolayer substance as determined by analysis attain the value for dibasic aluminium monomyristate.

Fig. 1 illustrates the relationship between the aluminium content of the monolayer substance and some quantities (A_0 and π_K) defining the monolayer. The quantities defining the monolayer on a substrate containing $1 \cdot 10^{-3}$ mole of aluminium sulphate per litre are plotted against the aluminium contents of the monolayer substance. It is seen from the curves that the properties of the monolayer undergo marked variation until the ratio of aluminium to myristic acid in the monolayer has increased to one mole of aluminium to three to two moles of myristic acid: A_0 diminishes to a minimum, and π_K rises to a maximum value and then decreases. The transformation of the monolayer substance from a substance containing one mole of aluminium to three to two moles of myristic acid to aluminium monomyristate, however, exerts only a slight influence on the monolayer properties; A_0 increases slightly and then remains practically constant, and π_K falls to a minimum value and then undergoes a slight increase.

The data that were considered characteristic of the aluminium myristate monolayer in Table 2, part A (for minimum A_0) in Part III of this series [1] hence apply to the monolayer containing one mole of aluminium to three to

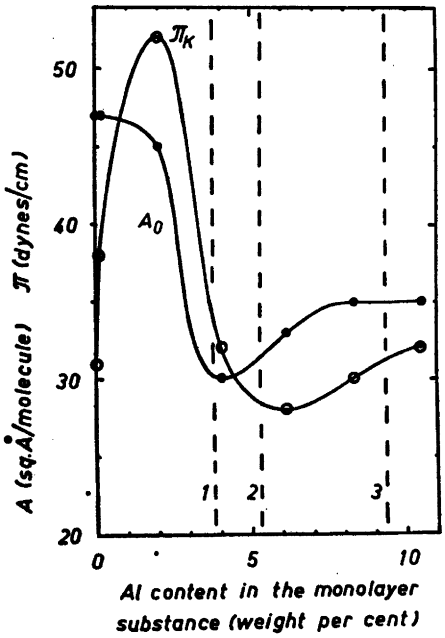

Fig. 1. The relation between the molecular area A_0 and the collapse pressure π_K and the aluminium content of the monolayer substance. 20° C. The dotted lines give theoretical aluminium contents for different aluminium myristates: 1 aluminium trimyristate, 2 monobasic aluminium dimyristate, and 3 dibasic aluminium monomyristate.

two moles of myristic acid and the data in part B of the same table (constant A_0) to a monolayer composed of aluminium monomyristate.

As in the case of the reaction of aluminium ions with a rosin acid monolayer [2], the final reaction product of the interaction between aluminium ions and a myristic acid monolayer is an aluminium soap in which the molar ratio of aluminium to acid is unity. Significant differences are, however, observed in other respects in the reactions of the two acids with aluminium ions. In the case of myristic acid the monolayer attains properties characteristic of the aluminium myristate monolayer already when the aluminium content has increased to one mole to three to two moles of myristic acid. In the case of rosin acid, properties typical of aluminium rosinate become evident only after the monolayer substance has been transformed into aluminium monorosinate.

The reasons for the differences in the reactions of these acids with aluminium ions in the substrate may be connected with the dimensions of the acids and the aluminium ions. The cross-sectional area of the rosin acid molecule is about twice as large as that of the myristic acid molecule. The coulombic forces of attraction due to the positive charges of the aluminium ions immediately below a densely packed monolayer can thus affect the carboxyl groups of a greater number of myristic acid molecules than of rosin acid molecules. It can be estimated that in the compressed monolayer the force of attraction acting on the carboxylic groups of three myristic acid molecules is about twice that on three or two rosin acid molecules. In addition, the great flexibility of the fatty acid molecules makes an intimate interaction between an aluminium ion and several myristic acid molecules easier also in the expan-

ded monolayers. The above-mentioned circumstances perhaps explain why the pressure-area curve typical of an aluminium soap is in one case attained already when the monolayer contains one mole of aluminium for three to two moles of myristic acid but in the other case only when the molar ratio of aluminium to rosin acid is increased to unity. Furthermore it should be noted that the hexaquo-aluminium ion is relatively large in size. The cross-sectional areas of this and of other possible aluminium ions are considerable greater than that of myristic acid molecule but somewhat smaller than that of the rosin acid molecule. Obviously these circumstances play a great part in effecting those changes which the monolayer properties undergo when the rosin acid and myristic acid monolayers are gradually transformed into aluminium rosinate or myristate monolayers and for the differences observed in the behaviour of the two acids in their reaction with the aluminium ions.

Acknowledgment. The work has been carried out in collaboration with and with the financial support of the *O.Y. Keskuslaboratorio-Centrallaboratorium A.B. (The Research Institute of Finnish Pulp and Paper Industries)*, Helsingfors, Finland.

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Some 3-Dialkylaminomethylindoles and 3,3′-Diindolylmethanes

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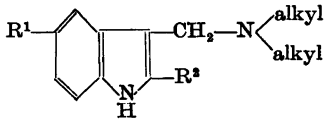
A number of 3-dialkylaminomethylindoles have been prepared by the Mannich reaction. In some cases 3,3′-diindolylmethanes were obtained as by-products. 3,3′-diindolylmethanes could also be prepared from an indole and formaldehyde or from an indole and a Mannich indole base. By the latter method unsymmetrical diindolylmethanes could also be prepared.

In the course of some studies in the indole series, the primary object of which was the synthesis of potential antagonists to serotonin (5-hydroxytryptamine), a number of new 3-dialkylaminomethylindoles were prepared which are reported in this paper.

The indoles, which were of type I (R^1 = H, CH_3, CH_3O, Cl; R^2 = CH_3, C_6H_5), were easily obtained by the Mannich reaction. In some cases when diethylamine was used as the amino component neutral by-products were isolated which were shown to be 3,3′-diindolylmethanes (II).



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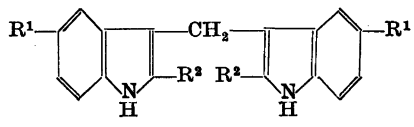
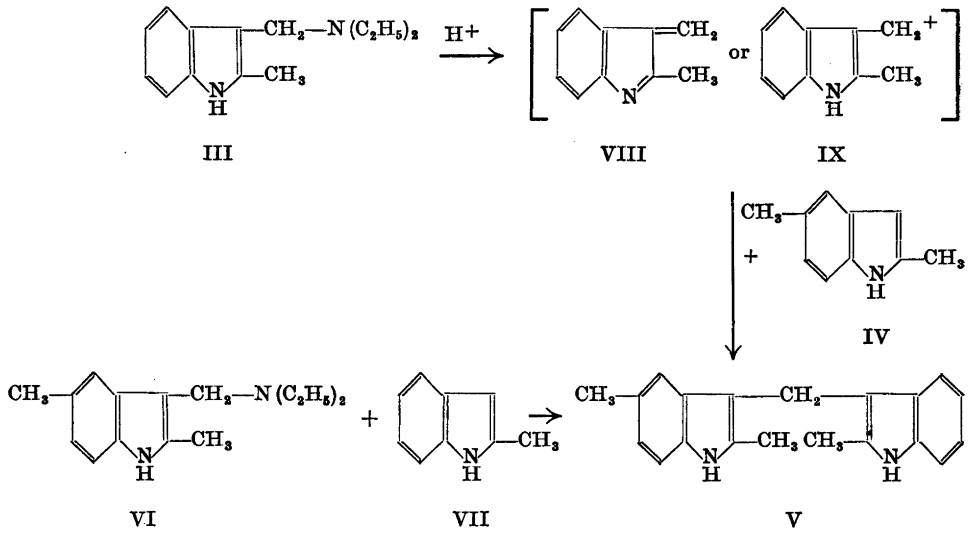


II

The formation of diindolyl compounds seemed to be promoted by insufficient cooling in the initial stage of the reaction. Small amounts of by-products have been reported to be formed in the Mannich reaction with indoles [1–5], but the constitutions of these compounds have only occasionally been subject to investigation [3,4]. However, diindolylmethanes have been found as by-products when tertiary Mannich indole bases and their quaternary salts were used as alkylating agents [6]. The diindolyl compounds could also be obtained from the starting indoles and formaldehyde. They were also prepared in good yields by the reaction between an indole unsubstituted in the 3-position and a Man-

nich indole base under the same experimental conditions as in the Mannich synthesis. Using the last named method unsymmetrical diindolylmethanes could also be obtained. Finally it was observed that salts of the Mannich bases gradually decomposed in aqueous solution giving diindolyl compounds.

The mechanism of the formation of the diindolylmethanes is not clear and may be different under different conditions. Their formation as by-products in the Mannich reaction may be due to a direct reaction between the starting indole and formaldehyde but they may also be formed by alkylation of the starting indole by the formed Mannich indole base. However, the formation of unsymmetrical diindolylmethanes is rather elucidative both with regard to the constitution of the product and to the reaction mechanism. Thus, the reac-



III VIII IX VI VII V

tion between 3-diethylaminomethyl-2-methylindole (III) and 2,5-dimethylindole (IV) affords the same product (V) as the reaction between 3-diethylaminomethyl-2,5-dimethylindole (VI) and 2-methylindole (VII). This seems to confirm that the indole nuclei are connected in 3,3′-position. If the reaction mechanism had been a reverse Mannich reaction followed by the reaction of the thus formed formaldehyde with an indole, the reaction product would most probably consist of a mixture of three different diindolyl compounds, two symmetrical and one unsymmetrical. It is more reasonable to adapt the mechanism proposed by Snyder and Eliel [7] for alkylations with gramine and its salts: Amine elimination from the Mannich base (III) gives an unsaturated intermediate (VIII) or a carbonium ion (IX) which reacts with an indole unsubstituted in the 3-position.

On the other hand, the formation of diindolyl compounds in aqueous solutions of salts of 3-diethylaminomethylindoles cannot be explained in this way, no indole unsubstituted in the 3-position being present. In this case it seems necessary to postulate the first step to be a reverse Mannich reaction followed

by recombination of the formaldehyde and the indole fragments or an alkylation of the indole with the Mannich base according to the mechanism outlined above.

The 3-dialkylaminomethylindoles have been investigated pharmacologically by Professor B. Uvnäs and his collaborators of the Department of Pharmacology, Karolinska Institutet, Stockholm. The results will be published elsewhere.

EXPERIMENTAL

3-Dialkylaminomethylindoles

2-Methylindole [8], 5-chloro-2-methylindole [9], 2,5-dimethylindole [10], 5-methoxy-2-methylindole [11], and 2-phenylindole [12] required as starting material were prepared according to procedures described in the literature.

3-Diethylaminomethyl-2-methylindole. To a cooled mixture of diethylamine (2.78 g, 0.038 mole), and 30 % formaldehyde (1.6 ml) in glacial acetic acid (2.5 ml) was added 2-methylindole (2.5 g, 0.019 mole) and the mixture was allowed to stand overnight at room temperature. Next day the orange solution was poured into water giving a solid product (A) which was filtered off. The filtrate was made alkaline with sodium hydroxide giving the desired Mannich base (2.1 g, 51 %); m.p. 89—90° after recrystallisation from 80 % methanol. (Found: C 78.3; H 9.40; N 13.05. $C_{14}H_{20}N_2$ requires C 77.7; H 9.32; N 12.95). The by-product (A) (1.2 g) was recrystallised twice from ethanol; m.p. 236—237.5°. Analysis indicated that the product had the composition $C_{19}H_{18}N_2$ (Found: C 82.9; H 6.52; N 10.2. $C_{19}H_{18}N_2$ requires C 83.2; H 6.61; N 10.2). Mixed m.p. with a sample of *2,2'-dimethyl-3,3'-diindolylmethane*, prepared from 2-methylindole and formaldehyde according to the directions of von Walther and Clemen [13], showed no depression.

By essentially the same procedure the following Mannich bases were prepared:

3-Pyrrolidinomethyl-2-methylindole. M.p. 139—140° (from ethanol). Yield 98 %. (Found: C 78.6; H 8.65; N 13.2. $C_{14}H_{18}N_2$ requires C 78.5; H 8.46; N 13.1).

3-Diethylaminomethyl-5-chloro-2-methylindole. M. p. 105—106° (from 80 % methanol). Yield 74 %. (Found: C 66.9; H 7.12. $C_{14}H_{19}ClN_2$ requires C 67.0; H 7.64).

As a by-product a small amount of *5,5'-dichloro-2,2'-dimethyl-3,3'-diindolylmethane*, m. p. 234—235° (from ethanol), was obtained.

3-Piperidinomethyl-5-chloro-2-methylindole. M. p. 165—166° (from ethanol). Yield 99 %. (Found: C 68.9; H 7.26. $C_{15}H_{19}ClN_2$ requires C 68.55; H 7.29).

3-Dimethylaminomethyl-2,5-dimethylindole. M. p. 160—162° (from ethanol). Yield 86 %. (Found: C 77.1; H 8.71. $C_{13}H_{18}N_2$ requires C 77.2; H 8.97). The synthesis of this compound has also been described in a recent paper by Qvadbeck and Röhm [14].

3-Diethylaminomethyl-2,5-dimethylindole. M. p. 107—108° (from ethanol). Yield 63 %. (Found: C 78.0; H 9.53. $C_{15}H_{22}N_2$ requires C 78.2; H 9.63).

As a by-product *2,2',5,5'-tetramethyl-3,3'-diindolylmethane*, m. p. 252—253° (from ethanol), was obtained. Yield 32 % calc. on 2,5-dimethylindole.

3-Piperidinomethyl-2,5-dimethylindole. M. p. 173—174° (from ethanol). Yield 89 %. (Found: C 79.6; H 9.19. $C_{16}H_{22}N_2$ requires C 79.3; H 9.15).

3-Pyrrolidinomethyl-2,5-dimethylindole. M. p. 167—168° (from ethanol). Yield 76 %. (Found: C 79.0; H 8.79. $C_{15}H_{20}N_2$ requires C 78.9; H 8.83).

3-Diethylaminomethyl-5-methoxy-2-methylindole. M. p. 84—85.5° (from 80 % methanol). Yield 73 %. (Found: C 72.5; H 8.75; N 11.2. $C_{15}H_{22}N_2O$ requires C 73.1; H 9.00; N 11.4).

3-Piperidinomethyl-5-methoxy-2-methylindole. M. p. 143—144° (from ethanol). Yield 97 %. (Found: C 74.4; H 8.64. $C_{16}H_{22}N_2O$ requires C 74.4; H 8.58).

3-Dimethylaminomethyl-2-phenylindole. M. p. 128—129° (from 80 % ethanol). Yield 93 %. (Found: C 81.1; H 6.98; N 11.1. $C_{17}H_{18}N_2$ requires C 81.6; H 7.24; N 11.2).

3-Piperidinomethyl-2-phenylindole. The *acetate* of this compound separated directly from the reaction mixture in 98 % yield; m. p. 157—158° (from benzene). (Found: C 75.1; H 7.88; N 8.00. $C_{22}H_{26}N_2O_2$ requires C 75.4; H 7.47; N 7.99).

The *base* was obtained on treatment of an aqueous suspension of the acetate with sodium carbonate; m. p. 117—118° (from 80 % methanol). (Found: C 83.0; H 7.90; N 9.34. $C_{20}H_{22}N_2$ requires C 82.7; H 7.63; N 9.64).

3,3'-Diindolylmethanes

2,2'-Dimethyl-3,3'-diindolylmethane. (*a*) This compound was obtained as a by-product in the reaction between 2-methylindole, formaldehyde and diethylamine as described above. It had earlier been prepared by von Walther and Clemen [13] and by Voisenet [15] from 2-methylindole and formaldehyde.

(*b*) It could also be obtained from 2-methylindole and 3-diethylaminomethyl-2-methylindole in the following way.

A mixture of 3-diethylaminomethyl-2-methylindole (0.25 g, 0.00115 mole), 2-methylindole (0.15 g, 0.00115 mole), and glacial acetic acid (0.3 ml) was heated on the water-bath for a few minutes until a clear solution was obtained. The mixture was then kept at room temperature for three hours. The separated crystals were collected (0.15 g, 48 %) and recrystallised from ethanol; m. p. 236—237°, undepressed on admixture with the product prepared according to von Walther and Clemen [13] and by the by-product obtained in the Mannich synthesis.

(*c*) An aqueous solution of 3-diethylamino-2-methylindole hydrochloride (0.5 %, pH 4) was kept at room temperature for 20 days. A small amount of solid material was gradually formed which was collected and recrystallised from ethanol; m. p. 236—237°, undepressed by the products described above.

2,2',5-Trimethyl-3,3'-diindolylmethane. The reaction of 3-diethylaminomethyl-2-methylindole was reacted with 2,5-dimethylindole in acetic acid was carried out as described under (*b*) above. M. p. 209—210° (from ethanol). Yield 39 %. (Found: C 82.8; H 6.96; N 9.47. $C_{20}H_{20}N_2$ requires C 83.3; H 6.99; N 9.71). This compound could also be prepared in the same way from 3-diethylaminomethyl-2,5-dimethylindole and 2-methylindole.

2,2',5,5'-Tetramethyl-3,3'-diindolylmethane. (*a*) This compound was obtained as described above as a by-product in the Mannich reaction between 2,5-dimethylindole, formaldehyde and diethylamine.

(*b*) A mixture of 2,5-dimethylindole (0.5 g), 30 % formaldehyde (0.3 ml), glacial acetic acid (0.4 ml) and ethanol (5 ml) was heated on the water-bath for a few minutes. The reaction product began to crystallise almost immediately. After cooling it was collected (0.4 g, 78 %) and recrystallised from ethanol-acetone (1:1); m. p. 252—253°, undepressed by the by-product from the Mannich reaction. (Found: C 82.9; H 7.56; N 9.47. $C_{21}H_{22}N_2$ requires C 83.4; H 7.33; N 9.26).

5-Chloro-2,2'-dimethyl-3,3'-diindolylmethane. Prepared in 96 % yield from 3-diethylaminomethyl-2-methylindole and 5-chloro-2-methylindole in acetic acid in the usual way; m. p. 180—181° (from ethanol). (Found: C 74.2; H 5.27; N 8.90. $C_{19}H_{17}ClN_2$ requires C 73.9; H 5.55; N 9.07).

5,5'-Dichloro-2,2'-dimethyl-3,3'-diindolylmethane. (*a*) A small amount of this compound was obtained as a by-product in the Mannich reaction between 5-chloro-2-methylindole, formaldehyde and diethylamine.

(*b*) It was also prepared in the usual way from formaldehyde and 5-chloro-2-methylindole in 48 % yield; m. p. 234—235° (from ethanol). (Found: C 65,8; H 4.99; N 8.17. $C_{19}H_{16}Cl_2N_2$ requires C 66.5; H 4.70; N 8.16).

2-Methyl-2'-phenyl-3,3'-diindolylmethane. Prepared from 3-diethylamino-2-methylindole and 2-phenylindole in 70 % yield; m. p. 205—207°, after recrystallisation from ethanol-acetone (3:1). (Found: C 85.4; H 6.09; N 8.08. $C_{24}H_{20}N_2$ requires C 85.7; H 5.99; N 8.32).

2,2'-Diphenyl-3,3'-diindolylmethane. Prepared from formaldehyde and 2-phenylindole. Yield 58 %; m. p. 184—185° (from ethanol). (Found: C 86.9; H 5.71; N 7.11. $C_{29}H_{22}N_2$ requires C 87.4; H 5.56; N 7.03).

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Studies on Local Anesthetics XII *

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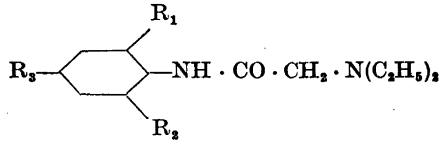
The N-(diethylaminoacetyl) derivatives of 2- and 4-fluoroaniline, 2-fluoro-4-toluidine, 4-fluoro-2-toluidine, 6-fluoro-2-toluidine and 4-fluoro-2,6-xylidine have been synthesized and tested for their local anesthetic action on the rabbit cornea. In comparison with xylocaine the compounds were found to have a lower activity and contrary to xylocaine they exercised an irritant action.

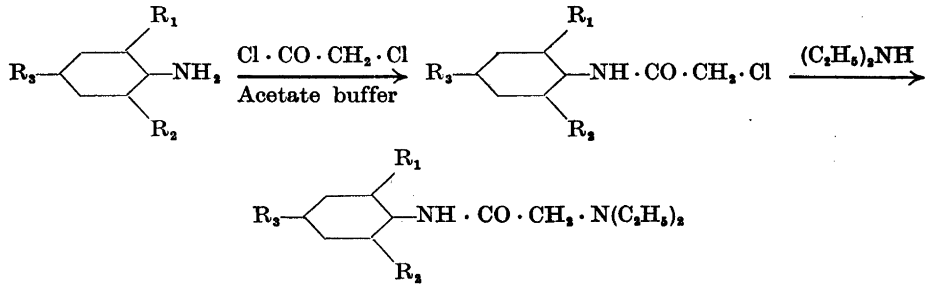
Six xylocaine analogues (I—VI), each with a fluorine atom in the benzene nucleus and which belong to the formula



	R_1	R_2	R_3
I:	F	H	H
II:	H	H	F
III:	F	H	CH_3
IV:	CH_3	H	F
V:	F	CH_3	H
VI:	CH_3	CH_3	F

have been synthesized and studied on their local anesthetic action.

The syntheses of the compounds were carried out according to the following scheme:



* For paper XI of this series see Löfgren and Tegnér [1].

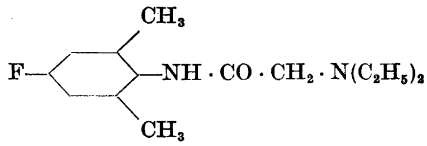
The necessary fluoroarylamines, $R_1R_2R_3{\equiv}C_6H_2.NH_2$, were produced by catalytical hydrogenation (Raney nickel) of the corresponding nitro derivatives. One of these nitro derivatives, 4-nitro-3,5-dimethyl-fluorobenzene, has not been prepared previously. It was made in the following manner: 3,5-Dimethylaniline was converted into 3,5-dimethylfluorobenzene by the Schiemann reaction. The fluoro derivative was then cautiously nitrated yielding a crystalline mixture which should consist of the two possible isomers, *viz.* 2-nitro- and 4-nitro-3,5-dimethylfluorobenzene. This mixture was subjected to a systematic fractional crystallization from methanol. In this way two substances, A and B, were obtained. They had about the same melting point value, but their crystalline forms differed distinctly. Further recrystallizations from a solvent mixture di-*n*-butyl ether — light petroleum 1:2 (v/v) did not alter the melting points. Product A, m.p. 56—57°, consisted of octahedral crystals, product B, m. p. 57—58°, of thin needles. A mixture of equal parts of A and B melted between 31° and 43°. The elementary analyses proved that each product corresponded to the composition of a mononitro-dimethylfluorobenzene. The yield of A was 46 % and that of B 14 %. By heating A with KOH in methanolic solution the fluorine atom was replaced by the methoxy group. The yield of anisole was high (78 %, calc. on recrystallized material). It should consist of either 2-nitro-3,5-dimethylanisole or the corresponding 4-nitro derivative. The melting point (51—52°) agreed with that reported [2,3] for 4-nitro-3,5-dimethylanisole. This compound was synthesized by way of nitration of 3,5-dimethylphenol and subsequent methylation of the 4-nitro derivative (Rowe *et al.*[2]). The mixed melting point showed no depression. If our anisole had consisted of the 2-nitro isomer (recorded [2,3] m. p. 45—46°), admixture with the 4-nitro isomer would have produced a large depression (*cf.* Wilds and Djerassi [3]). — Thus nitro derivative A was proved to be 4-nitro-3,5-dimethylfluorobenzene and consequently nitro derivative B was presumed to consist of 2-nitro-3,5-dimethylfluorobenzene.

The compounds I—VI were tested for their local anesthetic action on the rabbit cornea and compared with xylocaine *. In these tests the compounds gave durations of anesthesia which were inferior to that of xylocaine. Whith the exception of III which had about half the duration of that of xylocaine the other compounds gave durations equal to approximately one tenth of the xylocaine value. Further it was found that, contrary to xylocaine, all the compounds were irritating to the cornea.

Toxicity measurements, carried out with compounds V and VI by injections in white mice, gave LD_{50} values 0.88 g/kg and 0.60 g/kg, respectively. The corresponding value for xylocaine [4] is 0.34 g/kg.

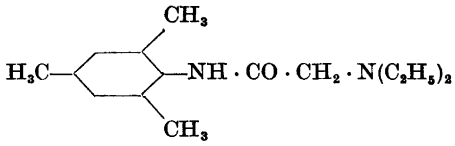
* For this purpose solutions containing 2 % of *base* and 0.9 % of NaCl were prepared by dissolving the base and NaCl in water under the addition of hydrochloric acid so that a pH value of 6.0 was obtained. The solutions were then compared on the cornea, using Wiedling's technique [4].

Since xylocaine is the non-fluorinated derivative of VI



VI

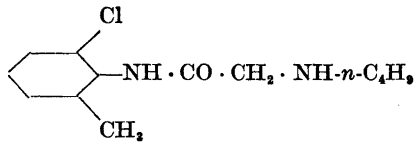
it might, perhaps, seem somewhat confusing that VI, in comparison with xylocaine, has such a low activity. If in VI, the fluorine atom is replaced by a methyl group, the resultant compound,



LL 31

known under the name of LL 31 (Löfgren [5], Löfgren and Lundqvist [6]), will have about the same effect on the cornea as has xylocaine (Wiedling [7]). In xylocaine there is a marked *ortho*-effect (Löfgren [8], Fischer and Löfgren [9]), the *ortho*-methyl groups being believed to interact with the electron pair of the amide nitrogen (Löfgren [8], *cf.* also Branch and Calvin [10]). This *ortho*-effect in xylocaine might be responsible for the outstanding physicochemical and physiological properties as compared with those of its nuclear isomers. In the LL 31 molecule there is also an *ortho*-effect (Löfgren [8]) though it very probably is less pronounced than that in xylocaine (*cf.* measurements of exaltations and ionization constants of xylocaine and related compounds[8]). The σ values of p-CH_3 and m-CH_3 are both negative whereas the corresponding values for fluorine are positive (*cf.* Hammet [11]). Thus in the vicinal grouping of LL 31 and VI, the electron pair of the nitrogen as well as the binding electrons in the two methyl groups are oppositely influenced, and therefore the strengths of the *ortho*-effects in the two molecules should be different and different from the *ortho*-effect in xylocaine. If, however, the *ortho*-effect in VI is weakened or strengthened relative to that of xylocaine, it becomes a question which can only be solved by further experiments.

A local anesthetic which differs from xylocaine in having a *n*-butylamino group instead of the diethylamino group and a chlorine atom instead of one of the two methyl groups is hostacain



Hostacain

which appeared some years ago on the market. According to Edlund and Wiedling [12] the compound is an irritant and has a duration on the rabbit cornea which is about one fifth of that of xylocaine. Replacement of the chlorine atom in hostacain by a methyl group leads to a compound, α-*n*-butylamino-2,6-dimethylacetanilide, which is an isomer of xylocaine and which was synthesized previously by Löfgren and Fischer [13]. The compound was found by Goldberg [14] to be a strong irritant and to exercise the same duration

as that of xylocaine on the rabbit cornea. Thus hostacain must have a shorter duration on the cornea than that of the xylocaine isomer and apparently chlorine, like fluorine (*cf.* above), cannot replace one of the *o*-situated methyl groups without causing a considerable decrease in the duration.

EXPERIMENTAL *

o-Fluoroaniline. The amine has previously been prepared by reduction of *o*-fluoronitrobenzene with iron and sulphuric acid [15] or with stannous chloride and hydrochloric acid [16].

Here the nitro compound — prepared according to Bennet *et al.*[17] by the Schiemann reaction — was hydrogenated in the presence of Raney nickel. Thus a solution of 21 g (0.14 mole) of *o*-fluoronitrobenzene in 100 ml of tetrahydrofuran and 2 g of moist catalyst were shaken with hydrogen at a pressure of about 2 atm. and a temperature of 50°. After about four hours no more hydrogen was consumed. The cooled solution was filtered from the catalyst and after removal of the solvent the residue was taken up in ether. The ethereal solution was exhaustively extracted with 1 N hydrochloric acid. To the combined aqueous extracts was added concentrated ammonia and the liberated base again taken up in ether. After drying the solution (Na_2SO_4), the solvent was driven off and the residue distilled *in vacuo*. The *o*-fluoroaniline was collected as an oil boiling at 55—56°/12 mm. Yield 7.3 g (0.066 mole), *i. e.* 44 %.

p-Fluoroaniline. *p*-Fluoronitrobenzene was prepared by nitration of fluorobenzene as described by Bradlow and VanderWerf.[18] This nitro compound was then hydrogenated catalytically under the same experimental conditions as given in the preparation of *o*-fluoroaniline (*cf.* above) with the only difference that absolute ethanol was used instead of tetrahydrofuran. The *p*-fluoroaniline was obtained as a colourless oil boiling at 84—85°/19 mm. The yield was 84 %. — The amine has previously been prepared by reducing the nitro derivative in various ways.[15,16,18,19]

α-Chloro-2-fluoroacetanilide and α-chloro-4-fluoroacetanilide. The compounds were synthesized by reaction between *o*-fluoroaniline or *p*-fluoroaniline and chloroacetyl chloride in an aqueous acetate buffer according to the general directions given by Löfgren [5,8] for this type of reaction.

α-Chloro-2-fluoroacetanilide: Thin, colourless needles from toluene; m. p. 89—90°; yield 68 %. (Found: C 51.1; H 3.83. Calc. for C_8H_7ClFNO (187.7): C 51.2; H 3.76).

α-Chloro-4-fluoroacetanilide: Colourless plates from 95 % ethanol; m. p. 129—130°; yield 85 %. (Found: C 51.4; H 3.94. Calc.: C 51.2; H 3.76).

α-Diethylamino-2-fluoroacetanilide (I) and α-diethylamino-4-fluoroacetanilide (II). A mixture of 12.0 g (0.0640 mole) of α-chloro-2-fluoroacetanilide or α-chloro-4-fluoroacetanilide, 12.2 g (0.167 mole) of diethylamine and 30 ml of dry benzene was refluxed for five hours. To the cool mixture were added 30 ml of dry ether and the solution was then filtered from the precipitated diethylammonium chloride. The filtrate was exhaustively extracted with 3 N hydrochloric acid and the combined extracts were made alkaline with concentrated ammonia. The liberated base was taken up in ether and after drying the solution (Na_2SO_4) the ether and some excess of diethylamine were evaporated. The residue was distilled under reduced pressure.

α-Diethylamino-2-fluoroacetanilide (I): Colourless oil of b. p. 104—105°/0.4 mm; $n_D^{20} = 1.5127$; yield 11.2 g (0.0499 mole), *i.e.* 78 %. (Found: C 64.2; H 7.72; Equiv.wt. 224. Calc. for $C_{12}H_{17}FN_2O$ (224.3): C 64.3; H 7.64; Equiv. wt. 224). — *Hydrochloride:* Colourless crystals from dioxan; m. p. 123—124°. (Found: C 55.1; H 7.10. Calc. for $C_{12}H_{18}ClFN_2O$ (260.7): C 55.3; H 6.96). — *Perchlorate:* Colourless, thin needles from absolute ethanol; m. p. 185—186°. (Found: C 44.4; H 5.63. Calc. for $C_{12}H_{18}ClFN_2O_5$ (324.7): C 44.4; H 5.59).

* M. p.'s corrected; b. p.'s uncorrected. The determination of equivalent weights on the synthesized bases was made by titrating them in 30 % ethanol with 0.1 N HCl; mixed indicator, methylene blue-methyl red.

α-Diethylamino-4-fluoroacetanilide (II): Colourless oil of b.p. 117—118°/0.3 mm; $n_D^{20} = 1.5163$; yield 12.3 g (0.0550 mole) *i. e.* 86 %. (Found: C 64.3; H 7.77; Equiv. wt. 224. Calc.: C 64.3; H 7.64; Equiv. wt. 224) — *Perchlorate:* Colourless crystals from absolute ethanol; m. p. 155—157°. (Found: C 44.5; H 5.65. Calc.: C 44.4; H 5.59).

2-Fluoro-4-methylaniline, 4-fluoro-2-methylaniline and 6-fluoro-2-methylaniline. First 3-fluoro-4-nitrotoluene, 5-fluoro-2-nitrotoluene and 3-fluoro-2-nitrotoluene were produced by nitration of 3-fluorotoluene, the procedure given by Schiemann [20] being followed. The nitro compounds were then reduced catalytically as described above in the preparation of *p*-fluoroaniline. Of the three anilines only 4-fluoro-2-methylaniline has been prepared previously (Schiemann [20]) and by reduction of the corresponding nitro derivative with tin and hydrochloric acid.

2-Fluoro-4-methylaniline: Colourless oil of b. p. 80—81°/14 mm; $n_D^{20} = 1.5355$; yield 66 %. (Found: N 11.0. Calc. for C_7H_8FN (125.1): N 11.2). — *Hydrochloride:* Colourless. thin needles from 95 % ethanol; m. p. > 300°; sublimation p. ~ 175°. (Found: Cl 22.1. Calc. for C_7H_9ClFN (161.6): Cl 21.9).

4-Fluoro-2-methylaniline: Colourless oil boiling at 94°/16 mm (the same b. p. as reported by Schiemann [20]); yield 75 %.

6-Fluoro-2methylaniline: Colourless oil of b. p. 88—89°/13 mm; yield 64 %. (Found: N 11.3. Calc.: N 11.2). — *Hydrochloride:* Colourless leaflets from 98 % ethanol; m. p. > 300°; sublimation p. ~ 170°. (Found: Cl 22.1. Calc.: Cl 21.9).

α-Chloro-2-fluoro-4-methylacetanilide, α-chloro-4-fluoro-2-methylacetanilide and α-chloro-6-fluoro-2-methylacetanilide. These anilides were all prepared by reaction between chloroacetyl chloride and the appropriate aniline according to the acetate buffer method given by Löfgren [5,8].

α-Chloro-2-fluoro-4-methylacetanilide: Colourless needles from 95 % ethanol; m. p. 132—133° yield 86 %. (Found: C 53.8; H 4.67. Calc. for C_9H_9ClFNO (201.6): C 53.6; H 4.50).

α-Chloro-4-fluoro-2-methylacetanilide: Colourless crystals from 95 % ethanol; m. p. 122—123°; yield 81 %. (Found: C 53.7; H 4.60. Calc.: C 53.6; H 4.50).

α-Chloro-6-fluoro-2-methylacetanilide: Colourless, thin needles from 95 % ethanol; m. p. 116—117°; yield 80 %. (Found: C 53.7; H 4.63. Calc.: C 53.6; H 4.50).

α-Diethylamino-2-fluoro-4-methylacetanilide (III), α-diethylamino-4-fluoro-2-methylacetanilide (IV) and α-diethylamino-6-fluoro-2-methylacetanilide (V). These bases were all synthesized from the corresponding α-chloro derivatives (see above) and diethylamine, applying the experimental conditions as described in the synthesis of α-diethylamino--2-fluoroacetanilide.

α-Diethylamino-2-fluoro-4-methylacetanilide (III): Colourless oil of b. p. 116—117°/0.4 mm; $n_D^{20} = 1.5133$; yield 81 %. (Found: C 65.5; H 8.06; Equiv. wt. 238. Calc. for $C_{13}H_{19}FN_2O$ (238.3): C 65.5; H 8.04; Equiv. wt. 238). — *Perchlorate:* Colourless, thin needles from absolute ethanol; m. p. 141—142°. (Found: C 46.1; H 6.11. Calc. for $C_{13}H_{20}ClFN_2O_5$ (338.8): C 46.1; H 5.95).

α-Diethylamino-4-fluoro-2-methylacetanilide (IV): Colourless oil of b. p. 134—135°/0.6 mm; $n_D^{20} = 1.5135$; yield 79 %. (Found: C 65.7; H 8.21; Equiv. wt. 239. Calc.: C 65.5; H 8.04; Equiv. wt. 238). — *Perchlorate:* Colourless needles from 95 % ethanol; m. p. 135—137°. (Found: C 46.2; H 6.14. Calc.: 46.1; H 5.95).

α-Diethylamino-6-fluoro-2-methylacetanilide (V): Colourless oil of b. p. 113—115°/0.2 mm; $n_D^{20} = 1.5155$; yield 72 %. (Found: C 65.5; H 8.07; Equiv. wt. 238. Calc.: C 65.5; H 8.04; Equiv. wt. 238). — *Perchlorate:* Colourless, thin needles from 95 % ethanol; m. p. 135—136°. (Found: C 46.2; H 6.16. Calc.: C 46.1; H 5.95).

3,5-Dimethylfluorobenzene. The compound was synthesized from 3,5-dimethylaniline by the Schiemann reaction.

The intermediate, 3,5-dimethylbenzenediazonium fluoborate, was made by adding fluoboric acid to the diazotised amine, the experimental conditions being the same as described by Roe [21] in the preparation of *m*-nitrobenzenediazonium fluoborate. Yield 91 %; decomposition p. 86°.

The salt was decomposed by following Roe's directions [21] for the production of *m*-fluorotoluene from *m*-toluenediazonium fluoborate. The desired 3,5-dimethylfluorobenzene was obtained as a colourless oil of b. p. 145°; $n_D^{25} = 1.4737$; yield 53 % (overall from amine). (Found: C 76.9; H 7.22. Calc. for C_8H_9F (124.2): C 77.4; H 7.31).

2-Nitro- and 4-nitro-3,5-dimethylfluorobenzene. In a 500 ml round-bottomed flask, provided with a mechanical stirrer, a dropping funnel and a thermometer, were placed 48.4 g (0.390 mole) of 3,5-dimethylfluorobenzene and 78 g of acetic anhydride. The flask was placed in an ice bath, and the mixture was cooled below 10°. Under stirring a solution of 36.9 g (0.586 mole) of fuming nitric acid ($d = 1.51$) in 23 g of glacial acetic acid and 23 g of acetic anhydride was added over a period of forty minutes, the temperature being kept between 15° and 20°.

When the addition of the nitric acid was complete, the reaction mixture was removed from the ice bath and allowed to stand at room temperature for two hours. The flask was then placed in a water bath and under shaking the mixture was kept at a temperature of 50° for ten minutes. The cooled reactants were poured slowly into 950 ml of ice-water and well stirred. Sodium chloride, 47 g, was added and the aqueous layer was decanted and extracted with 300 ml of ether. The ethereal extract was added to the residual nitro product, and the ethereal solution thus obtained was washed with 35 ml portions of a 10 % sodium hydroxide solution until the water extract was distinctly alkaline. The ether was then distilled off. At the end of this distillation the temperature of the water bath was kept not higher than 60° and as soon as practically no more ether distilled, heating was discontinued and the distilling flask cooled *. The residue, after the addition of 175 ml of 10 % sodium hydroxide solution, was steam-distilled (condenser with tap-water, receiver in ice-water). The pale yellow crystals were filtered off on a glass filter, drained as dry as possible and then further dried by keeping them in a non-evacuated desiccator, set aside in a cool place, over a mixture of equal parts of sodium hydroxide pellets and calcium chloride.

The dried crystalline material, which should consist almost entirely of the two possible isomers of mononitro-3,5-dimethylfluorobenzene, was subjected to a systematic fractional crystallization from methanol, applying the classical procedure (*cf.* for instance Morton [22]). As the result of these operations two pale yellow substances which differed distinctly as to their crystalline forms, but had about the same value of their melting points, were obtained. (Further recrystallizations of the two products from a solvent mixture di-*n*-butyl ether — light petroleum 1:2 (v/v) did not alter the melting points.) One of the two products (A) consisted of octahedral crystals of m. p. 56—57°; yield 30 g. This substance was predominant in the first crop of crystals, obtained from the methanolic solution. The other product (B) was made up of very thin needles melting at 57—58°; yield 9 g. A mixture of equal parts of A and B was found to melt in the range between 31° and 43°. Both compounds sublimed appreciably at room temperature and pressure, A more rapidly than B.

The elementary analyses gave evidence that each substance was a mononitro-3,5-dimethylfluorobenzene. (Found for A: C 56.5; H 4.80. Found for B: C 56.7; H 4.73. Calc. for $C_8H_8FNO_2$ (169.2): C 56.8; H 4.77).

Replacement of the fluorine atom by the methoxy group in A, proved it to be 4-nitro-3,5-dimethylfluorobenzene (*cf.* following section), so apparently B was identical with the other of the two possible isomers, *viz.* 2-nitro-3,5-dimethylfluorobenzene.

Determination of the position of the nitro group in mononitro-3,5-dimethylfluorobenzene A (*cf.* preceding section). A solution of 2.00 g (0.0118 mole) of mononitro-3,5-dimethylfluorobenzene A and 2.9 g (0.052 mole) of potassium hydroxide in 36 ml of methanol was refluxed for six hours. After cooling, the mixture was poured into 150 ml of water. A pale yellow precipitate formed. This was filtered off, washed with water and dried in a desiccator over silica gel. The melting point was found to be 49—50°; yield 1.90 g (89 %, assuming the product to be a nitro-3,5-dimethylanisole). The substance was recrystallized from ethanol under the addition of a little water. Thin, yellow needles melting at 51—52° were obtained; yield 1.7 g (79 %, *cf.* above). Another recrystallization from a

* The desired nitro derivatives are volatile and therefore a prolonged heating to remove the last traces of the ether should be avoided.

solvent mixture di-*n*-butyl ether — light petroleum 1:2 (v/v) did not alter the melting point which agrees with that of 4-nitro-3,5-dimethylanisole [2,3]. (Found: C 59.5; H 6.10. Calc. for $C_9H_{11}NO_3$ (181.2): C 59.7; H 6.12). 4-Nitro-3,5-dimethylanisole was prepared according to the method of Rowe *et al.*[2] (nitration of 3,5-dimethylphenol and subsequent methylation of the 4-nitro derivative). The mixed m. p. showed no depression. If the nitroanisole obtained from compound A had consisted of 2-nitro-3,5-dimethylanisole (recorded [2,3] m. p. 45—46°), admixture of the isomeric 4-nitro-3,5-dimethylanisole would have produced a large depression, such a mixture being an oil at ordinary room temperature (*cf.* Wilds *et al.*[3]).

4-Fluoro-2,6-dimethylaniline. This was made from 4-nitro-3,5-dimethylfluorobenzene by catalytical hydrogenation as described in the preparation of *p*-fluoroaniline. Colourless oil of b. p. 117—118°/14 mm; $n_D^{20} = 1.5352$; yield 72 %. (Found: N 10.3. Calc. for $C_8H_{10}FN$ (139.2): N 10.1. — *Perchlorate:* Colourless crystals from 98 % ethanol; m. p. 235° (decomp.). (Found: C 40.0; H 4.64. Calc. for $C_8H_{11}ClFNO_4$ (239.6): C 40.1; H 4.63).

α-Chloro-4-fluoro-2,6-dimethylacetanilide. The compound was prepared from chloroacetyl chloride and 4-fluoro-2,6-dimethylaniline according to the acetate buffer method given by Löfgren [5,8]. Thin, colourless needles from absolute ethanol; m. p. 193—194°; yield 91 %. (Found: C 55.6; H 5.17. Calc. for $C_{10}H_{11}ClFNO$ (215.7): C 55.7; H 5.14).

α-Diethylamino-4-fluoro-2,6-dimethylacetanilide (VI). This compound was made from diethylamine and α-chloro-4-fluoro-2,6-dimethylacetanilide in the same manner as described above in the synthesis of α-diethylamino-2-fluoroacetanilide. At the distillation the compound appeared as a colourless oil that soon solidified; b. p. 112—113°/0.1 mm; yield 85 %. After recrystallization from light petroleum the melting point was found to be 56—57°. (Found: C 66.7; H 8.46; Equiv. wt. 252. Calc. for $C_{14}H_{21}FN_2O$ (252.3): C 66.6; H 8.39; Equiv. wt. 252). — *Perchlorate:* Colourless, thin needles from 95 % ethanol; m. p. 179—180°. (Found: C 47.9; H 6.27. Calc. for $C_{14}H_{22}ClFN_2O_5$ (352.8): C 47.7; H 6.29).

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Conductivity of Sulphuric Acid in the 92—98 % Range

Determinations of Conductivity in Sulphuric Acid with and without Additions of Ferrous Sulphate

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By the erection of an ethylene-sulphuric acid-alcohol plant for *Aktieselskabet De Danske Spritfabrikker (The Danish Alcohol Factories Ltd)*, Denmark, the problem arose of determining acid strength of sulphuric acid with an accuracy of 0.2 % in the 92—98 % range. After consideration of the possibilities the conductivity was chosen as a measure for acid strength. As the values found in the literature displayed rather large variations a series of measurements were made in pure sulphuric acid having a strength of 92—98 %, and in acid saturated with ferrous sulphate, at 15, 20, and 25° C. Tables have been made for the relation between strength and conductivity at different temperatures. Above 94.5 % ferrous sulphate has a considerable influence on the conductivity. Mixtures of saturated and non-saturated acid have conductivities varying proportional with the contents of ferrous sulphate.

Determination of acid strength in strong sulphuric acid by means of measurement of specific conductivity is a method widely used, and several papers have been published on this subject. Also several instruments for measurement of conductivity with scales divided in per cent sulphuric acid are available.

The classical figures for specific electrical conductivity in sulphuric acid were measured by F. Kohlrausch [1], who determined the conductivity in the whole range 0—100 % together with the temperature coefficient. This work was shortly afterwards supplied by W. Kohlrausch [2] with measurements in 96—100 % and in oleum.

Knietsch [3] in a detailed paper mentions a multiplicity of physical constants for sulphuric acid, and among these also the resistance as determined in a special measuring vat. Dr. Bazlen, who had assisted in the work, has later on recalculated the figures found to yield figures for specific conductivity (*cf.* Abegg [4]).

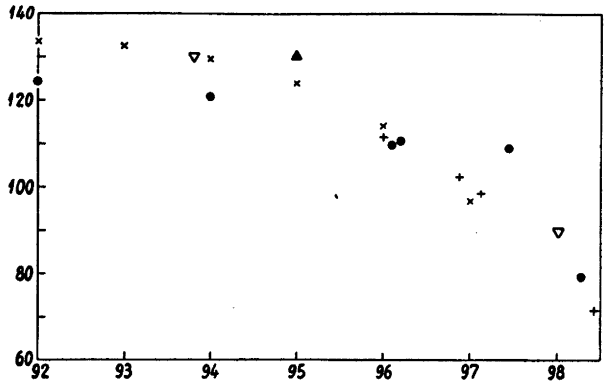

Fig. 1. Specific conductivity of sulphuric acid at 25° C.

× *F. Kohlrausch*
\+ *W. Kohlrausch*
● *R. Knietsch*
▽ *F. J. Symon*
▲ *J. E. Roughton*

Among more recent investigations may be mentioned those of Symon [5], who has determined the conductivity for three concentrations in the 93—99 % range and Roughton [6] in the 90—95 % range. The two last mentioned works deal substantially with conductivity at elevated temperatures. Finally Ust-Kachkintzev and Zhdanov [7] have determined the conductivity at temperatures from 20° C to 60° C in the concentration range from 9.9 to 97.5 % sulphuric acid. (Unfortunately it proved impossible to provide this last mentioned paper, the above has therefore been taken from Chemical Abstracts).

The above mentioned figures (excepting the Russian ones) have been plotted in Fig. 1 as specific conductivity at 25° C, the values given by F. and W. Kohlrausch being thereby corrected by means of the temperature coefficients given by the authors. It appears that there is some variation in the results.

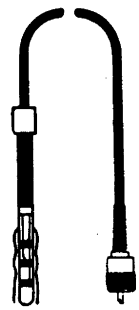

Fig. 2. The Immersion type cell CDC 104 (Courtesy Radiometer, Copenhagen).

APPARATUS AND MATERIALS

Apparatus. The determinations have been performed by means of a commercially available apparatus Radiometer Conductivity Meter, Type CDM 2, supplied with an immersion type cell CDC104 from the firm Radiometer in Copenhagen. The apparatus functions with a constant alternating voltage with a frequency of 3 000 cps.

The measurements have been made with the cell shown in Fig. 2. The electrodes consist of three interspaced platinum rings, the uppermost and nethermost of which are interconnected. In the measurements two individual cells have been used.

As a control a few measurements have been made in a cell consisting of 2 platinum plates with a surface of 1 cm^2 with an interspacing of 1 cm. The same values for the conductivity were found with both types of cells. The measurements were made in glass containers provided with thermometer, and the contents were kept at constant temperature within 0.1° C in a water thermostat.

Materials used. In preparing the mixtures of acid analytical grade sulphuric acid was used without further purification as a single measurement with redistilled acid did not show any differences due to the distillation.

The ferrous sulphate used was anhydrous, analytical grade, which was dried one night at 100°C before use.

The titrations have been performed with anhydrous, analytical grade sodium carbonate.

EXPERIMENTAL

A series of sulphuric acid mixtures having a concentration of 92 to 98 % were prepared by weighing.

Acid saturated with ferrous sulphate was prepared by addition of excess dried ferrous sulphate to the acid and standing for 2 days with frequent agitation. That the solutions were really saturated was secured by repeated measurements with an interval of 1 day, by which identical results were found.

Determination of acid strength

The accurate acid strength was determined by neutralization with sodium carbonate, at least two determinations being made on each acid sample.

About 2 g of acid were weighed exactly in a weighing bottle with cap. Due to the hygroscopic properties of the acid these weighings were performed as quickly as possible. In preliminary experiments it had been found that no uptake of water could take place under the circumstances used.

The sodium carbonate previously dried at 300° C was weighed in a platinum crucible and was dried for further 30 minutes before final weighing.

The acid was quantitatively transferred to a high glass beaker by means of a little water, and the sodium carbonate was added in small portions taking care that no losses due to spraying occurred. The carbon dioxide was removed by boiling, and the solution was thereafter electrometrically titrated to pH 7.0. The amounts of acid and carbonate were in each case adjusted to each other, so that less than 1 ml decinormal NaOH was required for the titration. The titration error could hereby be limited to within about 0.5 ‰.

In order to control the titrations a constant boiling sulphuric acid was prepared according to Kunzler [8]. The acid was prepared at a barometric reading of 762 mm Hg corresponding to 98.48 % by weight of sulphuric acid (all weighings corrected for the buoyancy caused by the air).

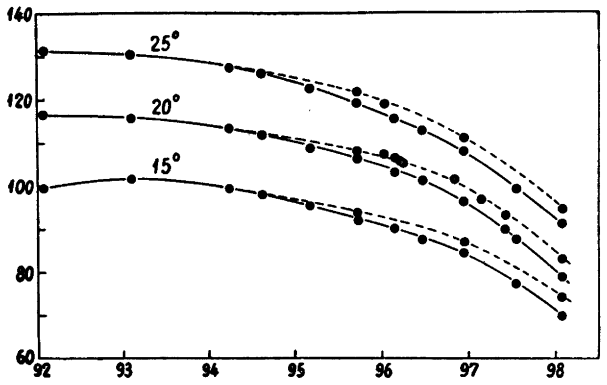

Fig. 3. Specific conductivity of sulphuric acid and of acid saturated with ferrous sulphate at 15, 20, and 25° C.
Full line: Sulphuric acid.
Dotted line: Sulphuric acid with ferrous sulphate.

Four titrations were made with this acid with the following results:

	98.49 %
	98.52 %
	98.49 %
	98.50 %
Average:	98.50 %

The first three titrations were made with the amounts mentioned above, while in the fourth titration ten times as much acid and carbonate was used. From the titrations of the constant boiling acid it was concluded that the method for checking acid strength was sufficiently accurate.

Measurement of electrical conductivity

The resistance capacity of the cell was determined by measuring the conductivity in a saturated solution of sodium chloride and in 1, 0.1, 0.02, and 0.01 *N* potassium chloride, the glass container with the test solution being thereby placed in the thermostat. The values for the conductivity of these solutions were taken from Handbook of Chemistry and Physics [9]. The scale readings of the apparatus were adjusted to correspond with the known conductivities of these solutions, and the adjustment of the apparatus was identical for all these solutions.

The measurements in sulphuric acid were performed as follows: the cells were rinsed with some of the acid to be measured, whereafter the cell filled with the acid was placed in the thermostat with thermometer directly in the cell. When temperature equilibrium was attained which took about 30 minutes,

Table 1. Specific conductivity of sulphuric acid
1 mS = 10^{-3} ohm^{-1} cm^{-1}
mS

	15°	16°	17°	18°	19°	20°	21°	22°	23°	24°	25°
92.0	99.2	102.7	106.1	109.6	113.0	116.5	119.4	122.4	125.3	128.3	131.2
2	99.8	103.1	106.4	109.7	113.0	116.3	119.3	122.2	125.2	128.1	131.1
4	100.3	103.5	106.7	109.8	113.0	116.2	119.1	122.1	125.0	128.0	130.9
6	100.8	103.9	106.9	110.0	113.0	116.1	119.0	122.0	124.9	127.9	130.8
8	101.2	104.1	107.1	110.0	113.0	115.9	118.9	121.8	124.8	127.7	130.7
93.0	101.5	104.3	107.2	110.0	112.9	115.7	118.7	121.6	124.6	127.5	130.5
2	101.6	104.4	107.2	109.9	112.7	115.5	118.4	121.4	124.3	127.3	130.2
4	101.4	104.2	107.0	109.7	112.5	115.3	118.2	121.1	124.0	126.9	129.8
6	101.1	103.9	106.7	109.4	112.2	115.0	117.9	120.8	123.6	126.5	129.4
8	100.7	103.5	106.3	109.0	111.8	114.6	117.5	120.4	123.2	126.1	129.0
94.0	100.2	103.0	105.8	108.6	111.4	114.2	117.1	119.9	122.8	125.6	128.5
2	99.6	102.4	105.2	108.0	110.8	113.6	116.5	119.3	122.2	125.0	127.9
4	98.8	101.6	104.4	107.2	110.0	112.8	115.7	118.5	121.4	124.2	127.1
6	98.1	100.9	103.7	106.4	109.2	112.0	114.8	117.7	120.5	123.4	126.2
8	97.2	100.0	102.8	105.6	108.4	111.2	114.0	116.8	119.6	122.4	125.2
95.0	96.2	99.0	101.8	104.6	107.4	110.2	112.9	115.7	118.4	121.2	123.9
2	95.2	98.0	100.8	103.6	106.4	109.2	111.9	114.6	117.3	120.0	122.7
4	94.2	97.0	99.8	102.6	105.4	108.2	110.8	113.5	116.1	118.8	121.4
6	93.2	96.0	98.8	101.5	104.3	107.1	109.7	112.2	114.8	117.3	119.9
8	92.1	94.9	97.6	100.4	103.1	105.9	108.4	110.9	113.4	115.9	118.4
96.0	91.0	93.7	96.5	99.2	102.0	104.7	107.1	109.5	112.0	114.4	116.8
2	89.8	92.5	95.2	97.9	100.6	103.3	105.7	108.1	110.4	112.8	115.2
4	88.5	91.2	93.8	96.5	99.1	101.8	104.1	106.5	108.8	111.2	113.5
6	87.1	89.7	92.3	94.9	97.5	100.1	102.4	104.7	107.1	109.4	111.7
8	85.6	88.1	90.6	93.0	95.5	98.0	100.3	102.7	105.0	107.4	109.7
97.0	83.9	86.3	88.6	91.0	93.3	95.7	98.0	100.4	102.7	105.1	107.4
2	81.8	84.1	86.3	88.6	90.8	93.1	95.4	97.7	100.1	102.4	104.7
4	79.5	81.6	83.7	85.9	88.0	90.1	92.4	94.8	97.1	99.5	101.8
6	77.1	79.1	81.1	83.0	85.0	87.0	89.4	91.7	94.1	96.4	98.8
8	74.2	76.1	78.0	79.9	81.8	83.7	86.1	88.5	90.9	93.3	95.7
98.0	71.2	73.0	74.8	76.6	78.4	80.2	82.7	85.2	87.6	90.1	92.6

the conductivity of the cell was measured, from which the specific conductivity was found.

In this way 13 mixtures of sulphuric acid were measured at 15, 20, and 25° C.

RESULTS

The results found have been plotted in Fig. 3, and smoothed curves drawn through the plotted points. From the curves the conductivity for each 0.2 % acid strength was read, and the conductivity was interpolated for the intermediate temperatures. The figures found in this manner are listed in Tables 1 and 2. By comparison of the values at 25° C on Fig. 1 with the curve found it will be seen that the values found correspond with those of F. Kohlrausch in the 92—98 % range.

Table 2. Specific Conductivity of sulphuric acid saturated with ferrous sulphate.
mS

	15°	16°	17°	18°	19°	20°	21°	22°	23°	24°	25°
94.4	98.8	101.6	104.4	107.2	110.0	112.8	115.7	118.5	121.4	124.2	127.1
6	98.1	100.9	103.7	106.4	109.2	112.0	114.8	117.7	120.5	123.4	126.2
8	97.4	100.2	103.0	105.8	108.6	111.4	114.2	117.1	119.9	122.8	125.6
95.0	96.8	99.6	102.4	105.2	108.0	110.8	113.6	116.4	119.3	122.1	124.9
2	96.1	98.9	101.7	104.6	107.4	110.2	113.0	115.8	118.6	121.4	124.2
4	95.3	98.1	100.9	103.8	106.6	109.4	112.2	115.0	117.8	120.6	123.4
6	94.6	97.4	100.2	103.1	105.9	108.7	111.5	114.2	117.0	119.7	122.5
8	93.8	96.6	99.4	102.2	105.0	107.8	110.5	113.2	115.9	118.6	121.3
96.0	92.8	95.6	98.4	101.2	104.0	106.8	109.4	112.0	114.6	117.2	119.8
2	91.8	94.6	97.4	100.2	103.0	105.8	108.3	110.8	113.3	115.8	118.3
4	90.7	93.5	96.3	99.0	101.8	104.6	107.0	109.4	111.9	114.3	116.7
6	89.5	92.2	95.0	97.7	100.5	103.2	105.5	107.8	110.2	112.5	114.8
8	88.2	90.9	93.5	96.2	98.8	101.5	103.7	105.9	108.2	110.4	112.6
97.0	86.7	89.2	91.7	94.3	96.8	99.3	101.5	103.7	105.8	108.0	110.2
2	84.8	87.2	89.6	92.0	94.4	96.8	99.0	101.1	103.3	105.4	107.6
4	82.7	85.0	87.2	89.5	91.7	94.0	96.2	98.4	100.5	102.7	104.9
6	80.4	82.6	84.7	86.9	89.0	91.2	93.4	95.5	97.7	99.8	102.0
8	77.9	79.9	81.9	83.9	85.9	87.9	90.1	92.3	94.6	96.8	99.0
98.0	75.3	77.2	79.0	80.9	82.7	84.6	86.9	89.2	91.4	93.7	96.0

From the curves on Fig. 3 it will be seen that the conductivity of sulphuric acid saturated with ferrous sulphate is higher than for the pure acid. An error of about 0.25 % in acid strength will be made by neglecting this effect in the 94.5—98 % range.

In order to examine this fact, the conductivity was measured in mixtures of acids of identical strength, one of which was saturated with ferrous sulphate and the other of which did not contain the salt. It was by these determinations found that the variation is practically proportional with the content of ferrous sulphate. Two experiments of this kind have been listed in Table 3.

Accuracy. The determination of the acid strength as above mentioned has been performed with an accuracy within 0.5 ‰.

Table 3. Specific conductivity of sulphuric acid with varying concentrations of ferrous sulphate.

	mS at20° C
Pure acid, 97.43 % by weight	90.1
» » 97.43 » » » 1/3 saturated	91.0
» » 97.43 » » » 2/3 »	91.8
» » 97.43 » » » , saturated	92.5
Pure acid, 96.21 » by weight	103.2
» » 96.21 » » » , 1/2 saturated	104.2
» » 96.21 » » » , saturated	105.5

The determination of the conductivity has for each acid mixture been performed in two different cells, and three readings have been made for each. The furnisher of the apparatus states an accuracy of 2 % in the range used, but as the results are mean values of six readings the accuracy of the measurements is substantially better.

The temperature has been read before and after each reading of the conductivity with an accuracy of less than 0.05° C.

The measurements all in all are believed to be accurate within 1 %.

Acknowledgments. The author wants to express his gratitude to *Aktieselskabet Dansk Gærings-Industri* for permission to publish this paper.

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Low-molecular Carbohydrates in Algae

X *. Investigation of *Furcellaria fastigiata*

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The red alga *Furcellaria fastigiata* has been investigated. *Meso*-inositol, D-mannitol, floridoside and a new glycoside, 3-floridoside α-D-mannopyranoside ** were isolated. An internal salt, di-*N*-methyltaurine, not found in Nature before, was also isolated.

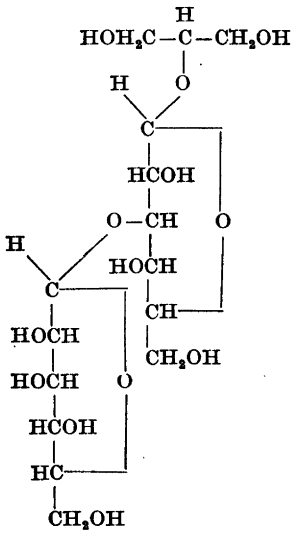
In previous papers in this series, investigations of several brown and some green algae have been reported. It could be expected that the red algae should differ considerably from these. The present paper records an investigation of the red alga *Furcellaria fastigiata*, *Florideae*, order *Gigartinales*. Four substances of carbohydrate nature were isolated, *meso*-inositol, D-mannitol, floridoside and a new glycoside, together with an internal salt.

Meso-inositol frequently occurs in the plant kingdom but the isolation of mannitol is more unexpected. Of the hexitols only dulcitol [1,2] and D-sorbitol [3] have been reported to occur in red algae. It is of course not excluded that the mannitol comes from impurities in the plant material, the relatively high amount (0.05 %), however, makes this assumption less probable. The glycerol galactoside, floridoside, first isolated by Colin and Guéguen [4], has been found in a great number of red algae. Thus all the algae of the order *Gigartinales* investigated by Augier and coworkers contained this substance. (Ref.[5] and preceding papers.) Putman and Hassid [6] recently showed it to be 2-glycerol α-D-galactopyranoside. In addition to floridoside (0.3 %) another glycoside (0.2 %) was isolated from *F. fastigiata*. It did not crystallise but was purified *via* its crystalline acetate, m. p. 153—154°, $[\alpha]_D^{20}$ 103° (in chloroform). It was non-reducing and on hydrolysis yielded galactose, mannose and glycerol in equimolecular proportions. As floridoside occurs in the same plant it was reasonable to assume that it was a mannoside of this substance. The molecular rotations of methyl α-D-galactopyranoside tetraacetate and methyl α-D-

* Part IX, *Acta Chem. Scand.* **9** (1955) 807.

** A note on the isolation and structure of this substance has been published in *Acta Chem. Scand.* **8** (1954) 869.

mannopyranoside tetraacetate are +38 800 and +47 800, respectively, and the sum of these figures, +86 600, agrees fairly well with the molecular rotation of the glycoside nonaacetate, + 82400, indicating that it is an α-mannoside. The glycoside consumed 2 moles of periodate, with the formation of 1 mole of formic acid, hence the mannose should be linked to the hydroxyl group at C_3 in the galactose unit of floridoside and the substance should have the structure I. This was also demonstrated by a Barry [7] degradation, *i. e.* treatment of the periodate oxidised glycoside with phenyl hydrazine, when the oxidised units are split off. Floridoside, identical with an authentic specimen, was obtained in a satisfactory yield (50 %) by this treatment, in agreement with the postulated structure.



I

Another α-mannoside, that of L-glyceric acid, recently showed to be 2-L-glyceric acid α-D-mannopyranoside (Part IX), has been isolated from several red algae [5]. This substance was not found in *F. fastigiata* but the presence of it in small amounts could not, however, be excluded. In addition to the substances isolated, the presence of glycerol, galactose and a number of unidentified substances was demonstrated by investigation of fractions from the carbon column separation by paper chromatography.

A substance of a different type was isolated from the first fractions of the carbon column separation. It had a high m.p., ~300° (decomp.), and was very soluble in water but insoluble in organic solvents. It was optically inactive and was not removed from an aqueous solution by filtering through a strongly acid and a weak basic ion exchange resin. Analyses were consistent with the formula $C_4H_9O_3NS$, and, further, an N-methyl analysis showed that two methyl groups were linked to the nitrogen. Of the rather few structures consistent with these facts, that of di-*N*-methyltaurine, $(CH_3)_2\overset{+}{N}HCH_2CH_2\overset{-}{S}O_3$, seemed most reasonable. This substance was synthesised, and the I.R. absorption spectra of the natural and the synthetic materials proved to be identical. The purification of the natural product involved considerable losses and it is possible that it was contaminated with some similar substances. Di-*N*-methyltaurine has not been isolated from natural sources before, but a somewhat similar substance, choline sulphate, $(CH_3)_3\overset{+}{N}CH_2CH_2O\overset{-}{S}O_3$, occurs in some fungi [8] and lichens [9]. The rôle of these substances in the plants is unknown but they may be intermediates in a transmethylation reaction.

EXPERIMENTAL

(Melting points uncorrected)

The alga (585 g) was continuously extracted with ether for 3 days and then with methanol for 14 days. The methanol extract was shaken with water and filtered. The aqueous solution was deionised by filtering through columns of Amberlite IR 120 and IR 4B and concentrated to a sirup (8 g). The acids from the IR 4B colum nwere eluted with aqueous ammonia and concentrated. This solution showed only a low positive rotation, indicating that it could not contain the α-mannoside glycerate in substancial amounts and was not further investigated.

The sirup (8 g) was dissolved in 1 % ethanol (50 ml) and added to the top of a carbon-Celite column (50 × 5 cm) and eluted, first with 1 % aqueous ethanol (4 000 ml), followed by aqueous ethanol, the concentration of which was continuously increased from 1 % to 30 %. The extract was divided into fractions which were investigated by paper chromatography and similar fractions were combined and concentrated to dryness.

The first fractions (1.7 g) consisted of the internal salt mixed with some substances of low R_F-values in the solvent mixture ethyl acetate-acetic acid-water, 3:1:1, which were developed with the silver nitrate-sodium ethoxide reagent. By recrystallising from aqueous ethanol a rather pure product (300 mg), m.p. 285—288° (decomp.) was obtained. On further recrystallisation the melting point was raised to 299—302°.

Part of the mother liquors from the first recrystallisations were concentrated to dryness and acetylated with a mixture of acetic anhydride and pyridine. The pyridine and excess of acetic anhydride were distilled off under reduced pressure and the residue partitioned between water and chloroform. The chloroform phase was dried over calcium chloride and coloured impurities were removed by filtering through a short column of aluminium oxide. The chloroform was distilled off and the residue crystallised from ethanol, yielding a small amount of *meso*-inositol hexaacetate (10 mg). M. p. 206—208°, undepressed on admixture with an authentic sample.

Paper chromatographic investigation of the next fraction (0.58 g) revealed the presence of a hexitol. After several recrystallisations from ethanol pure mannitol (300 mg), m. p. 163—164°, was obtained.

A later fraction (2.2 g) contained a substance chromatographically indistinguishable from floridoside. Crystallisation from ethanol yielded pure floridoside (1.7 g). $[\alpha]_D^{20}$ +163° (*c*, 2.0 in water). M. p. 127—128°, undepressed on admixture with authentic floridoside. Acetate, m. p. 100—101°.

The next fraction (1.05 g) contained a substance with the R_F-value of a disaccharide but was non-reducing and could not be developed with sugar reagents as anisidine phosphate. It did not crystallise but on acetylation yielded a crystalline acetate (1.5 g). M. p. 153—154°. $[\alpha]_D^{20}$ +103° (*c*, 2.0 in chloroform).

Di-N-methyltaurine. As the internal salt described above melted with decomposition, the m. p. was no criterium of its purity. An analytical sample was prepared by three further recrystallisations from aqueous ethanol of the product with m. p. 298—303°. (Found: C 31.4; H 7.22; N 9.40; S 20.9; (N)—CH_3 19.0. Calc. for $C_4H_9O_3NS$: C 31.4; H 7.26; N 9.16; S 20.9; (N)—CH_3 19.6.) The substance was neutral, optically inactive and not absorbed by strongly acid or weakly basic ion exchange resins.

Synthesis of di-N-methyltaurine. Sodium 2-bromoethanesulphonate (11 g) was dissolved in 33 % aqueous dimethylamine (200 ml) and the mixture kept at room temperature for 10 days. It was then concentrated to dryness under reduced pressure, dissolved in water and deionised by filtering through columns of Amberlite IR 120 and IR 4B. The water was distilled off under reduced pressure and the residue recrystallised from aqueous ethanol, yielding pure di-*N*-methyltaurine (5.4 g, 68 %), m. p. 299—304° (decomp.). The method is essentially the same as that previously used for the synthesis of this and similar substances, improved, however, by the use of ion exchange resins to remove the salts. The I. R. absorption spectra of the synthetic and natural products were identical.

3-Floridoside α-mannoside. In order to get further amounts of this substance another sample of the alga (1 700 g) was extracted and worked up as above, yielding the acetate (6.0 g), m. p. 153—154°, $[\alpha]_D^{20}$ + 103° (*c*, 2.0 in chloroform). (Found: C 49.6; H 5.52. Calc.

for $C_{33}H_{46}O_{23}$: C 49.9; H 5.85.) This was deacetylated catalytically to the amorphous glycoside, chromatographically identical with the original, unacetylated product and yielding the same acetate on subsequent acetylation, showing that the substance was not affected by the acetylation-deacetylation procedure. The presence of galactose, mannose and glycerol in the hydrolysate of the glycoside was demonstrated by paper chromatography and the proportions of these substances was determined as 0.99:0.92:1.00 by the method of Hirst and Jones [10]. A small amount of the glycoside (80 mg) was hydrolysed with 0.1 N hydrochloric acid (5 ml) at 100° for 24 hours. The acid was removed by ion exchange and the solution concentrated to 1 ml. Phenylhydrazine (100 mg) and acetic acid (0.25 ml) were added and the mixture was kept at room temperature for 2 hours. Mannose phenylhydrazone (24 mg) precipitated and was filtered off. M. p. 185—187°, undepressed on admixture with authentic material. Then methylphenylhydrazine (100 mg) was added to the filtrate. A precipitate was formed almost immediately and after two hours at room temperature the galactose methylphenylhydrazone was filtered off and recrystallised from ethanol. Yield, 19 mg, m. p. 184—185°, undepressed on admixture with an authentic sample. On oxidation with 0.1 M sodium metaperiodate at room temperature for 24 hours the glycoside consumed 1.8 moles of periodate and 0.87 moles of formic acid were formed.

Barry degradation of 3-floridoside α-mannoside. The acetylated glycoside (2.0 g) was dissolved in boiling anhydrous ethanol (25 ml) and a small amount of sodium ethoxide in ethanol added to the solution. After 3 hours the mixture was diluted with water (50 ml), filtered through a column of Amberlite IR 120 and concentrated to a sirup. The sirup was dissolved in a solution of sodium metaperiodate (1.2 g) in water (20 ml) and kept at room temperature for 24 hours. The solution was then deionised, using the Amberlite resins IR 120 and IR 4B. This proved to be inconvenient as the acids obviously reacted with the basic resin and the solution turned brownish red. The solution was then concentrated under reduced pressure to 20 ml and freshly distilled phenylhydrazine (1.5 ml) was added, followed by enough acetic acid to bring the phenylhydrazine into solution. The mixture was heated on the steam bath for one hour and after cooling, the precipitate was filtered off and the solution extracted with ether, deionised and concentrated to a sirup. Paper chromatographic investigation of this sirup revealed the presence of floridoside, while the floridoside mannoside had completely disappeared. Neither the sirup itself nor the acetylated sirup crystallised. Finally the product was deacetylated and purified by chromatography on a carbon column, yielding a colourless sirup (0.31 g) consisting of chromatographically pure floridoside. Crystallisation from ethanol yielded the pure substance (0.23 g), m. p. 126—127°, undepressed on admixture with authentic floridoside. The mother liquors were concentrated and acetylated, yielding pure floridoside hexaacetate (40 mg), m. p. 100—101°. The unnecessary acetylation-deacetylation steps involved some losses, and the yield of floridoside in the degradation could probably be increased.

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Low-molecular Carbohydrates in Algae

XI *. Investigation of *Porphyra umbilicalis*

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The low-molecular weight carbohydrates of the red alga *Porphyra umbilicalis* have been investigated. Two cyclitols, scyllitol and laminitol, two sugar alcohols, mannitol and volemitol and two glycosides, floridoside and a new substance *iso*floridoside, were isolated. The latter was shown to be 1-glycerol α-D-galactopyranoside.

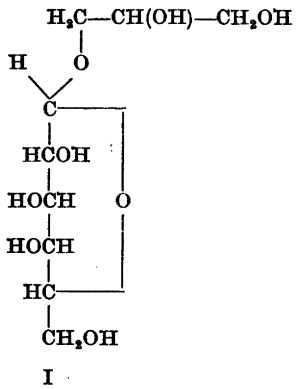
The red algae are botanically divided into two groups, *Bangioideae* and *Florideae*. It would be expected that there should be chemical differences between these groups. Most red algae previously investigated belong to *Florideae*, but the present paper records an investigation of an alga of the other group, *Porphyra umbilicalis*, order *Bangiales*, with respect to its low-molecular weight carbohydrates.

The two cyclitols scyllitol and laminitol were isolated. Scyllitol has not been found in algae before and has been isolated from only a few natural sources [1]. The other, laminitol, has recently been isolated from brown algae (Part VII [2]). It is a *C*-methyl inositol (Part VI[3]), probably with a *meso*-inositol configuration, although this has not yet been determined. We had believed it to be characteristic of brown algae, but the present isolation from a red alga indicates a more widespread occurrence.

Of the two sugar alcohols found, D-mannitol has recently been isolated from a red alga, *Furcellaria fastigiata*, order *Gigartinales*, *Florideae* (Part X) while D-volemitol has not been isolated from red algae before, but has been found in a brown alga, *Pelvetia canaliculata* (Part IV[4]). Thus of the polyols isolated from *P. umbilicalis*, laminitol, mannitol and volemitol are known to occur in brown algae. The possibility that they might come from impurities in the plant material could not be excluded. However the percentage of these substances present could be estimated as about 0.1 %, 0.03 % and 0.01 %, respectively, but in the brown algae investigated the percentage of mannitol is about 100 times higher than that of laminitol and it is therefore most improbable that the laminitol should come from small amounts of contaminating brown algae.

* Part X, *Acta Chem. Scand.* **9** (1955) 1093.

The alga also contained two glycosides, one with the same R_F-value as floridoside and the other slightly slower, although the order on the carbon column was reversed. It is our experience from work on the separation of carbohydrates, that of two isomeric or similar substances, the fastest on the paper is generally the slowest on the carbon column. The percentage of these two glycosides was rather high (3 %). They were not completely separated, but both were obtained in a pure state from extreme fractions. One, m. p. 124—126°, $[\alpha]_D$ +162°, was identical with floridoside. The other, m. p. 134—135°, $[\alpha]_D$+152°, on hydrolysis yielded glycerol and galactose, and the substance is consequently isomeric with floridoside. The high optical rotation indicates an α-galactosidic structure, and as floridoside is 2-glycerol α-galactopyranoside [5], the most probable alternative for the new substance should be 1-glycerol α-galactopyranoside (I).



I

The result of a periodate oxidation was in agreement with this assumption, the substance consuming 3 moles of periodate with the formation of 1 mole of formic acid. In this substance the glycerol is asymmetrically substituted, and thus there are two diastereoisomeric 1-glycerol α-galactosides, one of which has now been isolated in a pure, crystalline state. The configuration of the glycerol part has not been investigated, nor the question of whether one or both forms occur in the plant. This glycoside has not been isolated before. It is interesting to note, however, that Augier and du Mérac [6], when investigating the alga *Bangia atropurpurea*, also of the order *Bangiales*, obtained a sirup in a yield of 2.3 % which they believed to be floridoside and which on hydrolysis yielded galactose as single reducing sugar. Contrary to their expectation it did not crystallise, and in the light of the present investigation it appears most probable that their sirup consisted of a mixture of floridoside and *iso*floridoside. It seems therefore possible that the new glycoside is characteristic for the order *Bangiales* or the whole group *Bangioideae*.

An internal salt, probably similar to the di-N-methyltaurine from *Furcellaria fastigiata*, was also isolated and the presence of glycerol, galactose and several unidentified substances was indicated by paper chromatography.

EXPERIMENTAL

(Melting points uncorrected)

The alga (330 g) was extracted and worked up as previously described (Part X). The extract was deionised by filtering through columns of the ion exchange resins Amberlite IR 120 and IR 4B. The acids were eluted from the basic resin with aqueous ammonia and the solution was concentrated. It showed a low positive optical rotation, indicating the presence of little of any glyceric acid α-mannoside, known from other red algae. The deionised extract was concentrated to a sirup (19 g) which was then fractionated on a carbon-Celite column, using aqueous ethanol, the concentration of which was continuously increased, as eluent. Fractions were collected and investigated by paper chromatography. Whatman no. 1 filter paper, ethylacetate-acetic acid-water (3:1:1) and the silver nitrate-sodium ethoxide reagent were generally used. Similar fractions were com-

bined and, when necessary, refractionated on carbon columns or thick filter paper (Whatman 3 MM). The separation was quite complicated and will not be described in detail.

The first fraction (0.90 g), gave spots identical with those of scyllitol, laminitol and glycerol in addition to some unidentified. By crystallisation from water pure scyllitol (300 mg), m. p. ~350° alone or in admixture with an authentic sample, was obtained. By acetylation of the concentrated mother liquors further amounts could be isolated as scyllitol hexaacetate, m. p. 293–295°. By fractionation of some mother liquors on thick filter paper, a small amount of "internal salt" was separated from the carbohydrates. After recrystallisation from aqueous ethanol it melted at 300–315° (decomp.).

The next fraction (0.74 g) had a similar composition, the relative percentage of laminitol, however, was higher. By crystallisation from water, a fraction (270 mg) containing only scyllitol and laminitol was obtained. These were separated as the acetates, taking advantage of the very low solubility of scyllitol hexaacetate. Scyllitol hexaacetate (60 mg), m. p. 294–296°, and laminitol hexaacetate (150 mg), m. p. 151–153°, $[\alpha]_D^{20}$ −18° (*c*. 2.0 in chloroform) were obtained. The acetate of laminitol from *Laminaria cloustoni* [3] had m. p. 151–152° and $[\alpha]_D^{20}$ −19°, and a mixture of the two samples showed no depression of m. p.

The following fractions gave a rather complicated picture on the paper chromatograms. They were combined (2.6 g) and refractionated on a carbon column. Further amounts of scyllitol and laminitol were obtained in addition to mannitol (60 mg), m. p. 163–164° and volemitol (50 mg), m. p. 150–151° undepressed on admixture with authentic samples. The presence of galactose was indicated by paper chromatography.

*Iso*floridoside and floridoside did not separate well on the carbon column but from the first fraction containing these two substances (0.84 g), pure *iso*floridoside (0.41 g) was obtained by crystallisation from ethanol. M. p. 134–135°, $[\alpha]_D^{20}$ +152° (*c*, 2.0 in water). Analogously, from the last fraction (2.3 g), pure floridoside (1.2 g), m. p. 124–126°, $[\alpha]_D^{20}$ + 164° (*c*, 2.0 in water) was obtained. In total, 11.7 g of the mixed glycosides were isolated.

Isofloridoside. (Found: C 43.0; H 7.21. Calc. for $C_9H_{16}O_8$: C 42.5; H 7.14.) The substance was slightly slower than floridoside on the paper chromatogram and like floridoside, gave a rather weak spot with the silver nitrate-sodium ethoxide reagent. The hydrolysate gave two spots, indistinguishable from those of galactose and glycerol. A small amount (40 mg) was hydrolysed with 1 N hydrochloric acid (4 ml) at 100° for 20 hours, deionised and fractionated on thick filter paper. The components were eluted from the paper with water and the extracts concentrated to sirups. The sirup containing the slowest component was dissolved in water (0.5 ml), and acetic acid (5 drops) and methylphenylhydrazine (2 drops) were added. A precipitate was immediately formed. After 1 hour it was filtered off and recrystallised from ethanol, yielding galactose methylphenylhydrazone (8 mg), m. p. 184–185°, undepressed on admixture with an authentic sample. The sirup containing the fastest component was dissolved in 10 % aqueous sodium hydroxide (0.5 ml), benzoyl chloride (3 drops) was added and the mixture shaken vigorously for 5 minutes. After 30 minutes at 0°, water (0.5 ml) was added, the crystals formed were filtered off and recrystallised from 70 % ethanol. Yield, 6 mg, m. p. 71–72°, alone or in admixture with authentic glycerol tribenzoate. On oxidation with 0.1 M sodium metaperiodate at 35° for 12 hours, the substance consumed 3.00 ± 0.05 moles of periodate with the formation of 0.96 ± 0.05 moles of acid.

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Investigations on the Chemical Composition of Pollen from some Plants

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Pollen from *Zea mays* (two samples), *Alnus glutinosa*, *Alnus incana* and *Pinus montana* has been investigated. The pollen samples were collected directly from the plants and were of a very high degree of purity. The contamination with foreign pollen was not greater than 1 %.

Pollen from *Pinus montana* contained only 13 % protein, while the other pollen species contained about 25 %. The content of water-soluble components was also low in *Pinus montana* pollen. The contents of fats, ash, carbohydrates, sulphur and phosphorus were determined. The different samples showed considerable variations in these components.

The amino acid composition was investigated qualitatively by paper chromatography and 7 amino acids were also quantitatively determined by microbiological methods. No great difference between the pollen species could be shown either qualitatively or quantitatively (the amino acid content calculated as percent of the protein content).

The vitamin contents with respect to riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin and inositol were determined. The two samples of *Zea mays* proved to be rich in pantothenic acid and inositol, whilst pollen of the *Alnus* species had a high riboflavin content and a low inositol content. Pollen from *Pinus montana* was relatively low in pyridoxine.

The chemical composition of pollen has already been the object of several investigations. A short summary of the literature in this field was recently compiled by Lundén [1]. Most of the investigations have been made in connection with studies on the importance of pollen as bee food. In most cases, pollen carried by bees has been collected in pollen traps and the material investigated has thus consisted of mixtures of pollen from various plants. Only in a few cases has pollen from a single species been subjected to investigation, the pollen having been collected directly from the plants. Since it has been pointed out (*cf. e. g.* Maurizio [2]) that there are certain differences, with respect to bee food value, between pollen collected directly from the plants and pollen collected by bees from the same plants, there must occur either certain changes in the pollen collected by the bees or a mixing of the pollen with other

substances by the bees. In an investigation on the chemical composition of pollen, it is therefore preferable to use pollen collected directly from the plants.

The methods for collecting pollen have recently been improved so that considerable amounts of pure pollen can be taken from individual plants *. In the investigations described in the following, we have used pollen from four different plants, *viz. Zea mays*, *Alnus glutinosa*, *Alnus incana* and *Pinus montana*. All the samples were collected in 1954. In addition, pollen from *Zea mays* collected in 1953 was investigated in order to determine to what extent pollen from the same plant but from different years would show variations in the chemical composition. The two *Alnus* species were selected to determine whether two closely related species show any differences in their chemical composition. The investigation thus includes examples of monocotyledons, dicotyledons and conifers. (It is generally believed that pollen from conifers has a lower bee food value [2].) The pollen samples used had been dried, immediately after collection and purification, to a moisture content of 5—7 % and were kept cool and dry. The investigations were carried out during the autumn of 1954. The samples from 1954 had thus been stored only a few months and the samples from 1953 somewhat more than a year. In the case of the 1954 samples, any changes in the composition resulting from storage must be considered improbable, at least with respect to the substances investigated here.

The presence of other pollen species in the samples was determined by Professor Gunnar Erdtman, head of the Palynological Laboratory, Bromma, Sweden, and was found to be greatest in the samples of *Zea mays*, about 1 %. In the other samples, considerably less was found, only about 0.1 %. The presence of foreign pollen is so insignificant that it cannot affect the results of the analyses.

In our investigations we have performed certain general chemical analyses, investigated the amount of amino acids present and also determined the content of certain vitamins belonging to the B group.

GENERAL CHEMICAL COMPOSITION

For each of the pollen samples, determinations were made of the content of nitrogen (protein = N × 6.25), ash (sulphate ash), reducing sugars (as glucose), carbohydrates after acid hydrolysis (calculated as glucose), water-soluble components (extraction in Soxhlet apparatus) and lipoids (ether-extractable substances, Soxhlet). In addition, sulphur and phosphorus analyses were made. The values obtained are shown in Table 1.

Pollen from *Pinus montana* shows a low nitrogen content corresponding to approximately 14 % protein while the nitrogen contents in the other pollen species correspond to about 26 % protein. The lipoid content varies considerably, *e. g. Zea mays* pollen contains 2—5 % ether-soluble components while the *Alnus* species have a higher content (almost 10 %), *Pinus* pollen occupies an intermediate position in this respect. The sum of water-soluble and ether-soluble components is low for pollen from *Pinus montana*, which may possibly

* These methods are based on an invention by Mr. G. Carlsson at AB Cernelle, Vegeholm, Sweden.

Table 1.

	Zea mays 1953	*Zea mays* 1954	*Alnus glutinosa*	*Alnus incana*	*Pinus montana*
N %	4.1	4.2	4.1	4.2	2.2
Protein % (N × 6.25)	25.6	26.3	25.6	26.2	13.8
Sulphate ash %	4.9	4.9	2.4	2.8	3.0
P %	0.58	0.75	0.42	0.28	0.30
S %	0.43	0.30	0.24	0.32	0.18
Reducing sugars (as glucose) %	10.3	7.3	8.4	5.7	2.7
Total carbohydrates (as glucose) %	35.1	34.6	27.4	22.5	29.5
Water-soluble substances %	35.9	49.7	41.2	33.3	31.9
Ether-soluble substances %	5.0	1.8	9.4	13.2	7.1

be due to the higher content of resistant membrane substances (pollenine) in pollen from conifers [3].

Both the sulphur and the phosphorus contents show considerable variations. Especially, the large difference between the sulphur contents of the two samples from *Zea mays* should be observed. *Pinus montana* pollen has a low sulphur as well as phosphorus content. Sosa-Bourdouil [4] found in general a lower phosphorus content in gymnosperm than in angiosperm pollen.

AMINO ACIDS

In three of the samples, *viz. Zea mays* 1954, *Alnus glutinosa* and *Pinus montana*, the amino acid composition was investigated using paper chromatography. The pollen samples were hydrolyzed by boiling with 5 *N* hydrochloric acid for 16 h. The results of these analyses are shown in Table 2. In the chromatographical examination of pollen hydrolyzed with NaOH, tryptophane could not be detected. However, as the presence of this amino acid was demonstrated in the quantitative microbiological determinations (see below), it has been included in the table.

Table 2.

Amino acids	*Zea mays* 1954	*Alnus glutinosa*	*Pinus montana*
Alanine	+	+	+
α-Amino-butyric acid	+	−	−
Arginine	+	+	+
Aspartic acid	+	+	+
Cystine	+	+	+
Glutamic acid	+	+	+
Glycine	+	+	+
Histidine	+	+	+
Hydroxyproline	+	−	−
*Iso*leucine	+	+	+
Leucine	+	+	+
Lysine	+	+	+
Methionine	+	+	+
Phenylalanine	+	+	+
Proline	+	+	+
Serine	+	+	+
Threonine	+	+	+
Tryptohane	+	+	+
Tyrosine	+	+	+
Valine	+	+	+

The same eighteen amino acids have been found in each of the three pollen samples. In addition, the presence of hydroxyprolin and aminobutyric acid was demonstrated in pollen from *Zea mays*. Consequently, there seems to be no great difference in the amino acid composition.

In the case of certain amino acids, quantitative determinations were made by microbiological methods (Table 3). The pollen samples were hydrolyzed by autoclaving at 120° for 7 hours with 5 *N* H_2SO_4. For the determination of tryptophane, the pollen was hydrolyzed in the same way with 5 *N* NaOH.

Table 3. Amino acid content of the pollen samples (g of amino acid per 100 g protein).

Amino Acid	*Zea mays* 1953	*Zea mays* 1954	*Alnus glutinosa*	*Alnus incana*	*Pinus montana*
Arginine	6.3	5.7	9.8	6.2	6.4
Leucine	7.6	5.6	6.0	7.1	6.5
Lysine	5.9	5.0	4.7	5.0	5.1
Methionine	1.6	1.6	1.4	1.6	1.5
Phenylalanine	2.9	2.3	2.3	3.0	2.1
Tryptophane	0.6	0.6	0.8	0.4	0.8
Tyrosine	1.9	1.9	1.7	1.9	2.1

No determinations of tyrosine have previously been made on pollen. As far as the other amino acids investigated here are concerned, two previous investigations have been carried out — one with pollen from *Zea mays* [5] and another with some bee-collected pollen species and a mixed pollen [6]. Our determinations agree well with these earlier investigations and the results indicate that no great qualitative or quantitative differences exist with regard to the amino acid contents of the pollen proteins. The amino acid content per gram of pollen is of course less in the case of *Pinus montana*, owing to the low protein content of this pollen.

VITAMINS

The following vitamins were determined: riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin and inositol. With regard to riboflavin, nicotinic and pantothenic acid, values from a number of previous investigations are available, mostly concerning pollen mixtures[7-10]. As to pyridoxine, biotin and inositol in pollen only a few investigations have been made [10,11]. In the case of inositol, no quantitative determinations have previously been carried out.

In our investigations, all determinations have been carried out by microbiological methods.

Riboflavin was determined with *Lactobacillus casei* according to Snell and Strong [12], although the test was performed with the help of the paper-disc method. Samples (5 g) were hydrolyzed by autoclaving at 120° for 60 min in 50 ml of 0.1 N HCl.

Nicotinic acid was determined with *L. arabinosus* according to Snell and Wright [13] using the paper-disc method. Samples (5 g) were hydrolyzed with 50 ml of 1 *N* HCl at 120° for 15 min. In this test the whole nicotinic acid complex is determined.

Pantothenic acid was determined with *L. arabinosus* according to Skeggs and Wright [14] using the paper-disc method. Pantothenic acid determinations present certain difficulties owing to the fact that the liberation of pantothenic acid often requires special extrac-

tion methods. From a series of experiments involving different sample treatments, it was found that boiling with water gave as high values as enzymatic extraction. Pearson [9] even states, with regard to his investigations on the pantothenic acid content of pollen, that this extraction method gives maximum values. Samples (5 g) of pollen were consequently extracted three times with 100 ml of water and the combined extracts obtained tested as described above.

Pyridoxine was determined with *Neurospora sitophila* according to Stokes *et al.*[15]. Samples (2.5 g) were hydrolyzed with 20 ml 1 *N* NaOH at 120° for 60 min. By this method the whole pyridoxine complex is determined.

Biotin was determined with *L. arabinosus* according to Lynes and Norris [16]. Samples (1 g) were hydrolyzed with 50 ml 3 *N* H_2SO_4 at 120° for 30 min according to Barton-Wright [17].

Inositol was determined partly with *Saccharomyces carlsbergensis* according to Atkin *et al.* [18] and partly with *Neurospora crassa according to Beadle* [19]. The samples were hydrolyzed by boiling with 50 ml 18 % HCl per gram of pollen at 120° for 6 h. The values obtained with both methods were well in agreement.

The results obtained have been summarized in Table 4. The values shown are the averages of 6—10 analyses.

Earlier determinations of the riboflavin content are derived from Vivino and Palmer [7], Ridi and Aboul Wafa [8] and Kitzes *et al.*[10] Ridi and Aboul Wafa investigated pollen from one single species, collected (probably directly) from the monocotyledon *Phoenix dactylifera*. They found the riboflavin content to be 6.5 μg/g, *i. e.* approximately the same as we have found for pollen from *Zea mays* and *Pinus montana*. The other two investigations [7,10] which were made on mixed pollen collected by bees, gave considerably higher values, *viz.* 16—19 μg/g pollen. In one of the reports [7], the main components of the pollen mixture are indicated. All of the plants were dicotyledons. In our investigation the two pollen samples from dicotyledons gave considerably higher values for the riboflavin content than the pollen from *Zea mays*. It is thus possible that pollen from monocotyledons contain smaller amounts of riboflavin than pollen from dicotyledons. The material studied, however, is not sufficient to serve as a basis for any general conclusions. As regards the riboflavin content in conifer pollen, no previous values are available.

The nicotinic acid content is fairly similar in the different pollen samples with exception of *Zea mays* pollen from 1953 where the nicotinic acid content is only about half that of the other samples. This low content may be due to the relatively long storage period and it is therefore intended that the analyses on the 1954 samples are to be repeated after one year in order to determine the influence of storing.

In the earlier investigations, Ridi and Aboul Wafa [8] found approximately the same values for pollen from *Phoenix dactylifera* as we have found in our pollen samples. Investigations made on pollen collected by bees [7,10] showed considerably higher values for the nicotinic acid content.

The pantothenic acid content in the two samples from *Zea mays* is about double that in the others. Values reported from other investigations show variations from 16 to 51 μg/g.

In the case of pyridoxine, only one previous investigation has been reported, apparently made on pollen collected by bees. This mixed pollen had a pyridoxine content of 9 μg/g.

The biotin content is very similar for the different pollen samples, about 0.6 μg/g. Only one determination [10] has previously been carried out and it showed that mixed pollen contained 0.25 μg/g.

Quantitative determinations of the inositol content have not previously been made. That inositol is present in pollen is indicated in an investigation by Anderson and Kulp [11] who isolated inositol from *Zea mays* pollen. This investigation showed that the content of free inositol is probably about 10 mg/g. We have determined the sum of free and bound inositol and our values for the inositol content in *Zea mays* pollen, 30 mg/g, are therefore not necessarily at variance with Anderson and Kulp's result. Pollen from *Zea mays* has a much higher inositol content than pollen from the other plants examined. Pollen from *Pinus montana* also has a fairly high content of inositol.

As the above investigations indicate, the two pollen samples from *Zea mays* are very similar except as regards their nicotinic acid contents. Also the two pollen samples from the *Alnus* species show a great deal of similarity, while the pollen from *Pinus montana* diverges from all of the others. *Zea mays* pollen is characterized by very high contents of pantothenic acid and inositol. Pollen from *Alnus* has a high riboflavin content. Finally, *Pinus montana* pollen has a considerably lower pyridoxine content than the other pollen samples. In view of its content of pantothenic acid and inositol, *Pinus montana* pollen occupies a position between pollen from *Zea mays* and pollen from *Alnus*. With regard to riboflavin, previous investigations [7,8,10] as well as our own have shown low values for monocotyledons and high values for dicotyledons. There consequently seems to exist a certain relationship between the systematic position of the plants and the vitamin content of their pollen. The number of pollen species examined, however, is too limited to permit any definit conclusions.

It is remarkable that pollen from *Pinus montana* does not in general show a lower vitamin content than the other pollen species. The only vitamin which occurs in smaller amount is pyridoxine. As far as the other vitamins are concerned, the contents are not significantly lower than the average values for the other pollen species. As Table 1 indicates, pollen from *Pinus montana* has a very low content of protein and water-soluble components. The protein content is only half that of the other pollen species. If the vitamin content is expressed as percent of the protein content, then the pyridoxine content will be about the same as for the other pollen species while the other vitamin contents will be about double. The vitamin content is also high when expressed as percent of the water-soluble components.

Table 4. Vitamins in the pollen samples (μg/g pollen dry weight).

	Zea mays 1953	*Zea mays* 1954	*Alnus glutinosa*	*Alnus incana*	*Pinus montana*
Riboflavin	5.7	6.2	11.2	12.1	5.6
Nicotinic acid	40.7	71.8	82.7	82.3	79.8
Pantothenic acid	14.2	12.7	4.2	5.0	7.8
Pyridoxine	5.9	5.5	5.7	6.8	3.1
Biotin	0.52	0.55	0.65	0.69	0.62
Inositol (mg/g)	30	30	3.0	3.5	9.0

The authors wish to express their sincere gratitude to Professor Gunnar Erdtman for determining the purity of the pollen samples. For the performance of the analyses summarized in Tables 1 and 2 we wish to express our sincere thanks to Dr. B. Sandberg, head of the Analytical Laboratory of AB Kabi, and to Dr. E. Sandegren, head of the Central Laboratory of AB Stockholms Bryggerier. The technical assistance of Miss S. Bobeck, Miss C. Jacobsson and Mr. R. Härdén is gratefully acknowledged. The pollen samples used in these investigations were supplied by AB Cernelle, Vegeholm, Sweden.

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The Crystal Structure of Mo_5Si_3 and W_5Si_3

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The structure of W_5Si_3 has been determined using single crystal data. The elementary cell is body-centered tetragonal with 4 formula units in the cell. The space-group is D_{4h}^{18} $-I4/mcm$. The tungsten atoms occupy one 16-fold and one 4-fold position while the Si-atoms occupy one 8-fold and one 4-fold position.

The relations between the W_5Si_3-structure and the $D8_8$- and Fe_3P-structures are discussed.

The silicides of the transition metals have been extensively investigated in the recent years. A review of the structures and properties of these substances has been given by Nowotny and Parthé [1]. As was mentioned in an earlier paper [2] some attention has been devoted to these silicides at this institute, also, and in particular to the phases "Mo_3Si_2" and "W_3Si_2", the existence of which was first reported by Brewer and collaborators [3].

From powder photographs Schönberg [4] found the elementary cell of these phases to be bodycentered tetragonal with the dimensions $a = 9.62$ Å, $c = 4.90$ Å (Mo_5Si_3) and $a = 9.64_5$ Å, $c = 4.97$ Å (W_5Si_3).

A determination of the density of the Mo-phase gave the value of 8.2. This indicated that the composition of the phases should be Mo_5Si_3 and W_5Si_3 with four formula units in each elementary cell. There is a conspicuous similarity between these cells and that of Fe_3P, and one reason why this investigation was started was to see whether or not this similarity depended on any deeper resemblances between the two structures.

In a footnote in the above mentioned review [1] it is reported that the cell dimensions have also been determined by H. Schachner, who has found the additional isomorphous phases "Cr_3Si_2" and "Nb_3Si_2".

In the following this structure type is referred to as the "W_5Si_3 type" because the single crystal data were obtained from a crystal of W_5Si_3.

EXPERIMENTAL

The silicides were prepared from weighed amounts of silicon and transition metal by sintering in a carbon tube furnace and melting in an arc furnace in an atmosphere of argon. Powder photographs were taken in a camera of the Guinier type, using Cu$K\alpha$-

radiation. Good single crystals of the tungsten phase were obtained in the sample melted in the arc furnace. A single crystal of suitable size was picked out and Weissenberg photographs were taken around the *c*-axis, using Mo*K*-radiation. The layer lines 0—5 were recorded. The intensities were visually estimated. The Patterson-sections $P(xy0)$ and $P(xy1/4)$ and the preliminary Fourier projection $\varrho\ (xy)$ were computed on the Hägg-Laurent machine [5]. The final Fourier projection in which higher wave numbers were used than this machine could handle was made with the aid of the Beevers-Lipson strips.

DETERMINATION OF THE STRUCTURE

As *hkl*-reflexes were only observed when $h + k + l = 2n$ and $0kl$-reflexes only when $k = 2n$ and $l = 2n$, the possible space-groups were $D_{4h}^{18} - I4/mcm$, $C_{4v}^{10} - I4cm$ and $D_{2d}^{10} - I\bar{4}c2$.

Judging from the vector concentrations in the Patterson-space, D_{4h}^{18}—$I4/mcm$ * was considered to be the most probable one. A closer analysis of the $P(xyz)$ function showed that by placing 16 W_I in 16(k) with $x = 0.074$ and $y = 0.223$ and 4 W_{II} in 4(b) the positions and relative heights of all the strong vectors could be explained. Good agreement was also obtained between observed and calculated intensities.

Space considerations showed that the only Si-positions that would give reasonable interatomic distances were 8 Si_I in 8(h) with $x = 0.17$, and 4 Si_{II} in 4(a). The shortest Si_I—W_I vector would in that case have a very high multiplicity and it is indeed found in the Patterson-space with the expected relative height.

Finally, the Fourier projection $\varrho(xy)$ was evaluated (Fig. 1). An empirical correction for absorption and thermal movement has been applied. This correction did not change the parameters of the W atoms but gave a somewhat more even background. It is seen from the projection that there are small but evident indications of maxima where Si-atoms are expected to be situated. The final atomic positions are **.

				x	*y*	*z*
16	W	in	16(k)	0.074	0.223	0
4	W	in	4(b)	0	1/2	1/4
8	Si	in	8(h)	0.17	0.67	0
4	Si	in	4(a)	0	0	1/4

THE STRUCTURE

It is well known [1,3,6] that the structures of the transition metal silicides and borides reveal a strong tendency to form nonmetal-nonmetal bonds. This tendency is clearly manifested in the W_5Si_3-structure where the Si_{II}-atoms form straight chains with a Si-Si distance of 2.48 Å parallel to the *c*-axis. The fact that Si-Si bonds have been created here may be the reason why W_5Si_3 crystallizes in this structure and not in the $D8_8$-structure [7], which

* Quite recently this space-group has also been suggested by E. Parthé *et al.*[11]

** This structure is identical with that given in Ref.[2], but a space group of higher symmetry has been chosen.

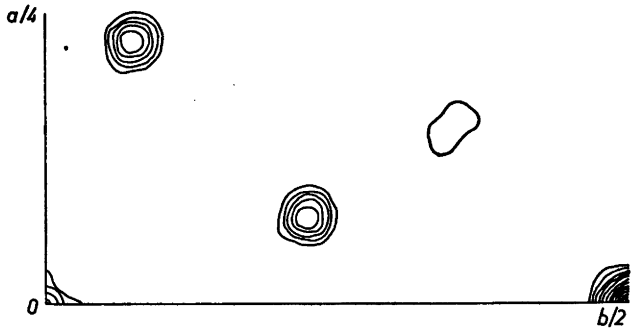

Fig. 1. Final Fourier projection ϱ (xy). Contours are drawn at intervals of approximately 40e Å^{-2} (around the W-atoms 80e Å^{-2}). 1/8 of the unit cell is shown.

corresponds to the same composition. There are, however, certain similarities between the two structure types.

As is seen from Table 1, where the environment of each atom is given, the coordination numbers of the metal atoms are the same in the two structures, but that of Si has increased by one in W_5Si_3. The characteristic straight chains of metal atoms with very close contact which are found among the silicides with the $D8_8$- and W_3O^8-structure are also found here. Fig. 2 shows two cells taken from the W_5Si_3-structure in order to illustrate the straight chains mentioned and their environment.

The degree of close-packing is slightly greater in W_5Si_3, the volume occupied by each atom being 1—2 % less than in the $D8_8$-structure.

Thus, there are common features in the two structures and the differences in lattice energy between them is probably small. When Mo and Si were sintered in a carbon tube furnace (~ 1 800°) approximately equal amounts of the ternary Mo-Si-C phase [9,10] ($D8_8$-structure) and the Mo_5Si_3-phase were obtained

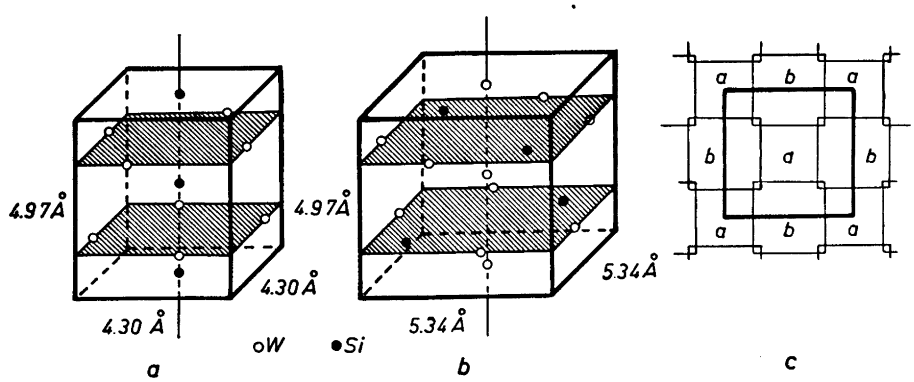

Fig. 2. a. Surroundings of a Si_{II}-chain. b. Surroundings of a W_{II}-chain. c. A projection on the basal plane of the cells a and b showing their relative positions in the unit cell.

Table 1.

	Interatomic distances in Å	Environment of the corr. atom in the $D8_8$-str.
W_I — 9 W	2.77; 2.86(2); 3.20(2); 3.21(2); 3.03(2)	Me_{II} —10 Me
— 6 Si	2.5; 2.6 (3); 2.8 (2)	— 5 Si
W_{II} —10 W	2.48 (2); 3.03 (8)	Me_I — 8 Me
— 4 Si	2.6 (4)	— 6 Si
Si_I —10 W	2.5 (2); 2.6 (4); 2.8 (4)	Si — 9 Me
(— 2 Si	3.2 (2).)	(— 2 Si)
Si_{II} — 8 W	2.58 (8)	Si — 9 Me
— 2 Si	2.48 (2)	(— 2 Si)

(only 0.2—0.3 % by weight of carbon was found in the sample). However, when annealed *in vacuo* (400°—1 100°) a complete transformation to the tetragonal phase occurred. The stabilizing effect of nonmetals with small atomic radius on the $D8_8$-structure has been discussed in the Refs.[1,9,10].

No significant similarities between Fe_3P and W_5Si_3 have been found.

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Constitution of Resin Phenols and their Biogenetic Relations

XIX *. The Structure of Sesamolin, the Configuration of Sesamin ** and the Nature of Fagarol ***

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The constitution of sesamolin has been elucidated and found to be the acetal (I). Sesamin has been shown to possess a "symmetrical" configuration (VII a or VIII a). The optically inactive fagarol of Priess from *Fagara xanthoxyloides* (Lam.) was obviously *d,l*-sesamin. A renewed investigation of the constituents of the bark of this species showed that it contains *d*-sesamin together with some *l*-sesamin.

In many countries it is prescribed by law that sesame oil must be added to oils and fat employed in the manufacture of margarin and artificial cream. This is due to the fact that sesame oil gives a very characteristic colour reaction (Baudouin's reaction: a red colour with hydrogen chloride and furfural) which makes it easy to differentiate between natural butter and cream and their artificial substitutes.

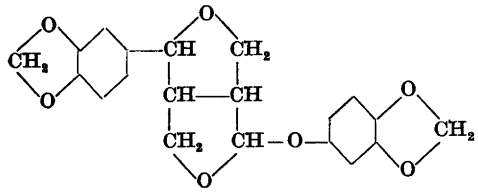
Baudouin's reaction is due to the presence in the oil of a compound m. p. 94°, isolated by the Italian chemists Malagnini and Armanni [1]. Their analyses indicated a formula $C_{20}H_{18}O_7$ or $C_{17}H_{16}O_6$. Under the influence of acids this compound furnishes sesamol (4-hydroxy-1,2-methylenedioxybenzene [1-3]). It is this substance which reacts with furfural to give the red colour in Baudouin's reaction.

Adriani [3] called the compound m. p. 94° sesamolin and showed that it had the elementary composition $C_{20}H_{18}O_7$ and this has now been confirmed. Adriani also showed that sesamolin on acid cleavage gives in addition to sesamol a new compound called samin according to the equation $C_{20}H_{18}O_7 + H_2O = C_7H_6O_3$ (sesamol) + $C_{13}H_{14}O_5$ (samin). Both sesamolin and samin are dextrorotatory ($[\alpha]_D$ *ca.* +220°, and *ca.* +100°, respectively). This cleavage of

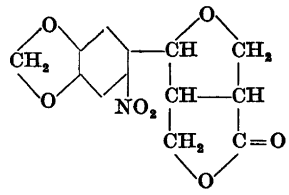
* Part XVIII *Acta Chem. Scand.* **8** (1954) 1827.
** Preliminary communication, *Chemistry & Industry* **1955** 567.
*** Preliminary communication, *Chemistry & Industry* **1955** 570.
**** On leave of absence from the *Scientific Department, Israeli Ministry of Defence.*

sesamolin indicates that the reaction is a simple hydrolysis and it has already been suggested by Böeseken, Cohen and Kip [4] that sesamolin might have a glycosidic structure.

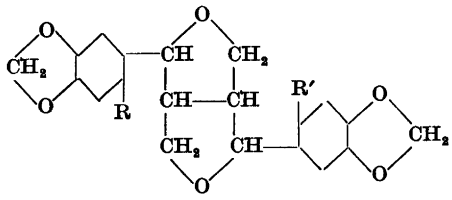
Sesame oil also contains another compound, sesamin (II) ($C_{20}H_{18}O_6$) differing from sesamolin only in possessing one oxygen atom less. Since both sesamolin and sesamin contain methylenedioxy groups the acetal structure (I) immediately suggests itself as the most probable structure for sesamolin.

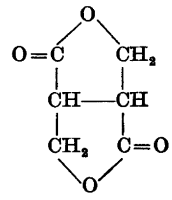


I



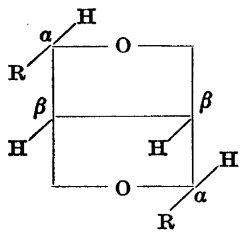
III



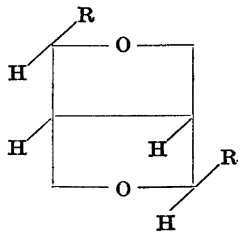


VI

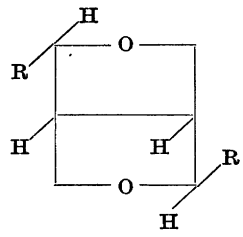
II R=R′=H
IV R=NO_2; R′=Br
V R=R′=Br
X R=NO_2; R′=H
XI R=Br; R′=H



VII a, b



VIII a, b



IX

VII a, VIII a: R=3,4-methylenedioxyphenyl

VII b, VIII b, IX: R=3-methoxy-4-hydroxyphenyl

The correctness of this assumption has been shown by converting both sesamolin and sesamin to the same nitrolactone $C_{13}H_{11}NO_7$ (III). This compound is obtained in excellent yield by cautious nitration of sesamolin and by the action of nitric acid on bromonitrosesamin (IV).

Sesamin belongs to the group of bis-furanoid lignans which can be divided into two subgroups, one containing methylenedioxy groups involving the oxygen atoms at 3,4,3′,4′ and another containing methoxyl groups (or hydroxyl groups) in these positions. The former includes sesamin, *iso*sesamin, *l*-sesamin and asarinin (*epi*-*l*-sesamin) and the latter pinoresinoldimethylether, *epi*pinoresinoldimethylether, eudesmin and *epi*eudesmin. Sesamin, *l*-sesamin, asarinin, pinoresinol and eudesmin have been isolated from natural sources. A β-glucoside of *epi*pinoresinolmonomethylether and a mono-β-glucoside of the optical antipode of *epi*pinoresinol (phillyrin (forsythin) and symplocosin, respectively) have also been encountered in nature. By elimination of the methylene groups followed by methylation, members of the former group have been transformed into compounds belonging to the latter group. (For references *cf.* Erdtman [5]).

Cohen [6] found that dibromosesamin (V) when treated with nitric acid furnished 4-bromo-5-nitromethylenedioxybenzene and Erdtman and Gripenberg [7] showed that dibromopinoresinoldimethylether similarly gives an excellent yield of 4-bromo-5-nitro-veratrol and the dextrorotatory bishydroxymethylsuccinic acid dilactone (VI). The same dextrorotatory dilactone has now been obtained from dibromosesamin. The optical activity of this dilactone shows that the hydrogen atoms at the β-carbon atoms in sesamin and pinoresinol are *cis* in concordance with the results obtained by v. Bruchhausen and Gerard [8] and by Haworth and Woodcock [9] by means of catalytic hydrogenation.

Sesamin (*d*-sesamin) and *l*-sesamin are optical antipodes and are converted into *d*-*iso*sesamin and *l*-*epi*sesamin (asarinin) under the influence of acids. This is due to inversion at one of the α-carbon atoms. Similarly pinoresinoldimethylether (*d*-pinoresinoldimethylether) and eudesmin (*l*-eudesmin) are optical antipodes and are converted into *d*-*epi*pinoresinol dimethylether and *l*-*epi*eudesmin by acids.

It has been shown by Erdtman [10] and by Gripenberg [11] that pinoresinol (and eudesmin) is "symmetrical" and hence can be described by one of the possible formulae (VII b) and (VIII b). Gripenberg [12] showed that *epi*pinoresinol lacks such "symmetry" and therefore has the formula (IX). Similarly we have now shown that mononitration of *d*-sesamin followed by bromination yields the same bromonitro-*d*-sesamin as that obtained by monobromination followed by nitration. Since no isomeric monosubstitution derivatives were found this shows that *d*-sesamin like pinoresinol is "symmetrical" (VII a) or (VIII a) (or their mirror images), and that these substances are structurally and configurationally analogous (as well as *l*-sesamin and *l*-eudesmin). These results invalidate the conclusions drawn by one of us [13] from the remarkable similarity in the changes in optical rotations on dinitration of *l*-asarinin and *l*-eudesmin.

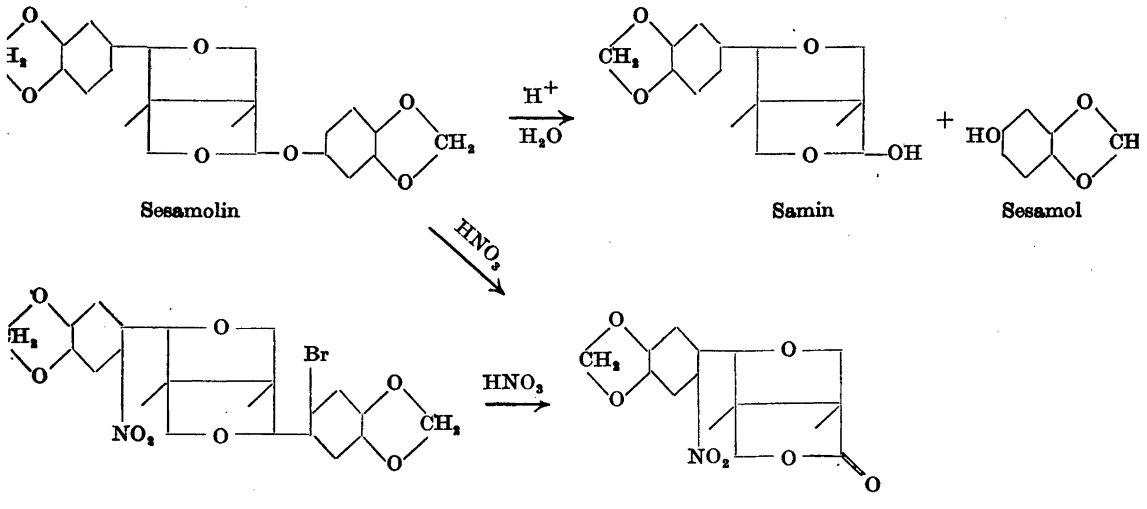
From the above chemical evidence the bisfuranoid keysubstances can be divided into two groups and this division is also confirmed by the molecular rotations.

The following chart, shows the relationships between the bisfuranoid keysubstances.

Group A, "symmetrical"		*Group B, "unsymmetrical"*
l-sesamin $[M]_D$ —234°	H^+	*l*-asarinin $[M]_D$ —422°
l-eudesmin $[M]_D$ —247°	H^+	*l-epi*eudesmin $[M]_D$ —560°
d-sesamin $[M]_D$ +242°	H^+	*d-iso*sesamin $[M]_D$ +422°
d-pinoresinoldimethylether $[M]_D$ +251°	H^+	*d-epi*pinoresinoldimethylether $[M]_D$ +545°

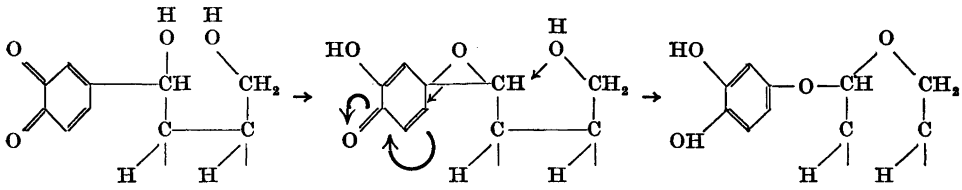
The only remaining configurational problem is the relative configurations at the α- and β-carbon atoms (*cf.* VII—IX). In group B the hydrogen atoms at the α-carbon atoms are *trans*. In group A they are *cis*, but it is not yet known whether they are *cis* or *trans* in relation to the hydrogen atoms at the β-carbon atoms.

As already mentioned, on treatment with nitric acid sesamolin gives a nitrolactone the composition of which is $C_{13}H_{11}NO_7$ and for which structure (III) was anticipated. When bromonitrosesamin is treated with nitric acid the brominated aromatic nucleus is extruded as 4-bromo-5-nitromethylene dioxybenzene and the lactone, $C_{13}H_{11}NO_7$, containing all the other carbon atoms of the sesamin molecule is formed. The yields of these fission products were almost quantitative and the reaction is directly analogous to the cleavage of 6,6′-dibromopinoresinoldimethylether or 6,6′-dibromosesamin (V). The lactones from sesamolin and from bromonitrosesamin were identical and this proves the structure of sesamolin and settles its configuration up to the limits set by our incomplete knowledge of the configuration of sesamin. The following formulae show the relationships between sesamin, sesamolin, samin and sesamol.



Sesamolin, obviously, is formed from sesamin or a nonmethylenated precursor by opening the molecule between one of the aromatic rings and the corresponding α-carbon atom.

The following formulae envisage a possible route but there are attractive alternatives including a rearrangement of a peroxide:



Among the lignans of unknown structure fagarol m. p. 127—128°, $C_{20}H_{18}O_6$, is unique in that it is optically inactive. It has been isolated from the root bark of *Fagara xanthoxyloides* Lam (Rutaceae), *cf.* Priess [14]. Östling [15] found a "sterol" $C_{27}H_{44}O$ m. p. 214° in the same material.

Recently Paris and Moyse-Mignon [16] reported the isolation of fagarol from *Fagara xanthoxyloides* and they comment upon certain discrepancies in the older literature and point out that they are probably due to confusion as regards the botanical identity of the (closely similar) *Fagara* species. Paris and Moyse-Mignon[17] have also isolated fagarol from the root bark of *Fagara viridis* A. Chev. Both species are West African (Senegal, Ivory Coast, Gold Coast, Togo).

Mameli [18], without adducing any new evidence, assumed that fagarol is *d,l*-cubebin ($C_{20}H_{20}O_6$) which, however, is not in agreement with the analyses. In view of the fact that the genera *Fagara* and *Xanthoxylum (Zanthoxylum)* are very closely related and that asarinin occurs in several *Xanthoxylum* species it appeared likely that fagarol might be identical with *d,l*-asarinin, m. p. 135—136°, or perhaps more likely with *d,l*-sesamin, m. p. 129—130°.

Through the good offices of Sir John Simonsen, London, we have been able to investigate bark from a tree identified by experts in London as *Fagara xanthoxyloides* and kindly provided by Dr. F. E. Hughes, Chief Conservator of Forests, Accra, Gold Coast. Extraction with light petroleum yielded an oil from which a compound m. p. 121—122° possessing the empirical composition $C_{20}H_{18}O_6$ and another compound m. p. 215°. Analysis showed the latter compound to have the formula $C_{30}H_{50}O$. It was obviously identical with Östling's "sterol" since it had the same optical rotation and also yielded an acetate with properties agreeing with those reported by Östling [15]. (Östling gives m. p. 119° instead of 219° obviously due to a misprint). The compound was identified (B. Thomas) as lupeol by direct comparison with an authentic sample kindly provided by Professor E. R. H. Jones, Manchester. Lupeol has been isolated from several *Fagara* and *Xanthoxylum* species [19,20].

The compound m. p. 121—122° was identical with *d*-sesamin. From the mother liquors of the sesamin, fractions were obtained which possessed the same or slightly higher melting points (123—124°) but lower rotations (about +50°). They did not depress the melting point of *d*-sesamin or *d,l*-sesamin and

obviously consisted of mixtures of *d*- and *l*-sesamin the *d*-form preponderating. According to Kaku and Ri [21] *d,l*-sesamin is a pseudoracemate ("Mischkristallisat") which is in agreement with our findings.

A specimen of "fagarol" ($[\alpha]_D$ +26° in chloroform) kindly provided by Professor Paris showed no depression on admixture with our sesamin preparations, but a large depression when mixed with *d,l*-asarinin. The IR absorption spectra of our sesamins (both that of *d*-sesamin and *d,l*-sesamin and the product of low optical rotation from *F. xanthoxyloides* and of the "fagarol" specimen of the French authors were identical. Hence Priess fagarol is certainly *d,l*-sesamin. The species *Fagara xanthoxyloides* is able to synthesise *d*-sesamin as well as *l*-sesamin although the relative amounts may perhaps vary from specimen to specimen. Alternatively there may be various strains of *Fagara xanthoxyloides* or hybrids between closely related species. It is possible that Priess recrystallised his fagarol until it consisted exclusively of *d,l*-sesamin, which has a higher melting point than the pure antipodes.

EXPERIMENTAL

Isolation of sesamolin and sesamin from sesame oil. Sesame oil was continuously extracted with methanol until the Baudouin reaction was negative or very faint. The methanol was evaporated and the residual oil treated with light petroleum (40–60°) and left in the refrigerator for 24 hours to crystallise. The crystalline material, judging by the rotation ($[\alpha]_D$ +115 ± 5°) was a mixture of sesamin and sesamolin in the approximate proportion of 5:1. By leaching with hot methanol the mixture was almost freed from sesamolin and the remaining sesamin was crystallised from ethanol giving pure sesamin, long rodlike needles, m. p. 122–122.5°, $[\alpha]_D^{20}$ +67.9°. The sesamolin was crystallised from methanol giving small leaflets m. p. 94–94.5°, $[\alpha]_D^{20}$ +212° (*c* 1.13 in chloroform). (Found: C 64.64; H 5.09. Calc. for $C_{20}H_{18}O_7$ (370.3): C 64.86; H 4.90).

Mononitrosesamin (X). Sesamin (2 g) was dissolved in a mixture of glacial acetic acid (15 ml) and acetic anhydride (5 ml). The solution was cooled to −10° and a cold solution of nitric acid (0.35 ml, d = 1.40) in acetic anhydride (3 ml) was added. After one hour water was added until the solution became cloudy and the dinitrosesamin which subsequently precipitated was collected (0.18 g) and crystallised from ethylacetate. Yellow-greenish needles, m. p. 240–241°, $[\alpha]_D^{20}$ +37° (*c* 1.10 in chloroform). When more water was added to the mother liquor an oil separated which solidified after a few hours. Fractional crystallisation from ethanol gave mononitrosesamin (0.85 g) as slightly yellow needles m. p. 142–143°, $[\alpha]_D^{20}$ −68.9° (*c* 1.42 in chloroform). (Found: N 3.86. Calc. for $C_{20}H_{17}NO_8$ (399.3): N 3.51). From the mother liquors sesamin (0.6 g) was recovered.

Monobromosesamin (XI). To a solution of sesamin (5 g) in pyridine (5 ml) and glacial acetic acid (50 ml) a solution of bromine (0.75 ml) in glacial acetic acid (30 ml) was added at room temperature and after 3/4 of an hour water was added until the solution became cloudy. Dibromosesamin (0.7 g) separated and was collected. Thin needles from ethanol, M. p. 180.5–181°, $[\alpha]_D^{20}$ −13.2° (*c* 1.01 in chloroform). Addition of more water to the mother liquor gave an oil which yielded monobromosesamin (1.72 g) and unreacted sesamin (0.5 g) on fractional crystallisation from methanol. The monobromosesamin crystallised in needles m. p. 85–85.5, $[\alpha]_D^{20}$ +29° (*c* 1.87 in chloroform). (Found: Br 18.98. Calc. for $C_{20}H_{17}BrO_6$ (433.3): Br 18.45).

Bromonitrosesamin (IV). A. Mononitrosesamin (0.5 g) dissolved in glacial acetic acid (8 ml) containing a few drops of pyridine was treated with bromine (0.06 ml) in glacial acetic acid (4 ml) at room temperature. After 15 minutes the product was precipitated in almost quantitative yield by the addition of water. The bromonitrosesamin crystallised from chloroform-ethanol in threadlike yellow needles, m. p. 201.5–202.5°

(softening at 186—187°), $[\alpha]_D^{20}$ −35° (*c* 1.35 in chloroform). (Found: Br 16.35. Calc. for $C_{20}H_{16}BeNO_8$ (478.3): Br 16.71).

B. Monobromosesamin (0.25 g) dissolved in a mixture of glacial acetic acid (1 ml) and acetic anhydride (1 ml) was nitrated at −15° with nitric acid (0.05 ml, d = 1.40) in acetic anhydride (1 ml). After a few minutes water was added to precipitate the product (0.21 g) which on crystallisation from chloroform-ethanol melted at 186—186.5°, $[\alpha]_D^{20}$ −34.5° (*c*. 1.34 in chloroform). When seeded with the higher melting bromonitrosesamin the melting point was raised to 201.5—202.5° and a mixed m. p. of preparations A and B showed no depression.

Degradation of dibromosesamin with nitric acid. Dibromosesamin (2 g) was added in small portions to nitric acid (30 ml, d = 1.40) at room temperature. A very faint reaction was noticed and a yellow crystalline precipitate was formed immediately and identified as 4-bromo-5-nitromethylenedioxybenzene (1.2 g). Yellow plates, m. p. 89° from ethanol. Addition of water to the nitration mixture gave another crop (0.27 g) of this compound. The mixture was then neutralised with bicarbonate, the water evaporated, and the dried residue continuously extracted with ether for 24 hours. The ether extract was evaporated and the residue dissolved in water, filtered and again evaporated to dryness. The product (0.25 g) was crystallised from benzene containing a few drops of ether. M. p. 162.5—163°, $[\alpha]_D^{20}$ +219° (*c* 0.89 in water). Erdtman and Gripenberg [7] found for the dilactone of bishydroxymethylsuccinic acid, m. p. 160—161°, $[\alpha]_D$ +206° (*c* 1.06 in water). Mixed m. p. of the two samples 160—162°.

Degradation of sesamolin with nitric acid. When sesamolin (0.3 g) was added to nitric acid (7 ml, d = 1.40 at room temperature it immediately dissolved with evolution of heat and nitrous fumes. After a few minutes water was added until the solution became cloudy and the mixture after leaving for some hours, deposited a yellow solid (0.16 g) which was crystallised from ligroin containing a little acetone. Thin yellow needles, m. p. 155.5—156.5°, $[\alpha]_D^{20}$ ±0° (*c* 0.68 in chloroform), +23.5° (*c* 0.53 in pyridine) (Found: C 53.14; H 3.87; N 5.11. Calc. for $C_{13}H_{11}O_7$ (293.2): C 53.24; H 3.78; N 4.78.)

Degradation of bromonitrosesamin with nitric acid. Bromonitrosesamin (0.6 g) was added to nitric acid (15 ml, d = 1.40) at room temperature. After 15 minutes the mixture was filtered from bromonitromethylenedioxybenzene (0.1 g) and then water was added until no more material was precipitated. The precipitate (0.44 g) was collected after standing some hours in the refrigerator and was found to be a mixture of bromonitromethylenedioxybenzene and another substance (0.27 g) which was crystallised from ligroin. M. p. 156—157°, undepressed on mixing with the degradation product from sesamolin.

Isolation of sesamin and lupeol from Fagara *bark.* The ground trunk or root bark of *Fagara xanthoxyloides* (Lam) was continuously extracted with light petroleum (40—60°) for 5 days. The residual oil after evaporation of the solvent was mixed with methanol. On standing crystals deposited. The first crops (A) were recrystallised from ethanol and further crops (B) from acetic acid. From A a relatively small amount of pure *d*-sesamin was obtained. M. p. 121—122°, $[\alpha]_D^{20}$ +65.2° (*c* 1.6 in chloroform). (Found: C 67.88; H 5.20. Calc. for $C_{20}H_{18}O_6$ (354.3): C 67.79; H 5.12). From the mother liquors fractions were obtained which showed a lower rotation but a slightly higher melting point, *e. g.* m. p. 123—124°, $[\alpha]_D^{20}$ + 49.8° (*c* 1.09 in chloroform). The melting points of these fractions were always between that of *d*-sesamin and *d,l*-sesamin. From all these sesamin fractions dibromo-*d*-sesamin was obtained m. p. 174—175°, $[\alpha]_D^{20}$ −14.3° (*c* 1.7 in chloroform), mixed m. p. with authentic dibromo-*d*-sesamin, $[\alpha]_D^{20}$ −15.0° (*c* 0.88 in chloroform), 175—176°. Cohen [6] gives $[\alpha]_D$ −9.6° (in chloroform) and Kaku and Ri [21] give $[\alpha]_D$ +16.2° (*c* 0.86 in chloroform) for dibromo-*l*-sesamin.

From B a colourless product was obtained by recrystallisation from acetic acid. M. p. 215—216°, undepressed by an authentic specimen of lupeol, $[\alpha]_D^{20}$ +25° (*c* 1.2 in chloroform). The acetate was prepared using acetic anhydride in pyridine and crystallised from chloroform-alcohol. M. p. and mixed m. p. 218—219°, $[\alpha]_D^{20}$ +30°. (*c* 1.2 in chloroform). (Found: C 81.86; H 11.23; Calc. for $C_{32}H_{52}O_2$ (468.7): C 81.99; H 11.18).

We are indebted to *Statens Tekniska Forskningsråd* and to the *Scientific Department, Israeli Ministry of Defence*, for financial support and to *Margarinbolaget*, Stockholm, for a generous gift of sesame oil.

Added in proof: The structure of sesamolin has recently been proved independently by M. Beroza (*J. Am. Oil Chemists' Soc.* **77** (1955) 3332) and by E. Haslam and R. D. Haworth (*J. Chem. Soc.* **1955** 827).

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The Infrared Absorption of the Different Crystal Forms of Some Normal Fatty Acids

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The infrared spectra of the different crystal forms of the same normal fatty acid are found to be different from each other. The parts of the spectra between 7.7 and 8.5 μ (1 300 and 1 180 cm^{-1}) and near 11.0 μ (900 cm^{-1}) are the best for identification purposes. In some cases the absorption near 5.9 μ (1 700 cm^{-1}), associated with the C=O stretching vibration, has been found to consist of two close peaks.

Sinclair *et al.*[1] and Bellamy [2,p.149] have found that a considerable frequency shift occurs for the OH out-of-plane deformation when passing from the unstable crystalline B-form to the stable crystalline C-form of *n*-stearic acid. In connection with crystal structure investigations of the different polymorphs of normal fatty acids, using single crystal and powder X-ray methods [3-7], the infrared absorption between 5 and 13 μ (2 000 cm^{-1} and 770 cm^{-1}, respectively) of the different crystal forms has been measured, in order to find if there are any other differences. The acids investigated were *n*-pentadecanoic acid, *n*-palmitic acid and *n*-stearic acid.

MATERIAL USED

The very pure acids used have been prepared by Professor E. Stenhagen and his collaborators. The melting points of the *n*-pentadecanoic acid, *n*-palmitic acid and *n*-stearic acid are 52.3° C, 62.9° C and 69.7° C, respectively.

There are three polymorphs of acids with an even number of carbon atoms and three of acids with an odd number of carbon atoms [3].

The A′-form of *n*-pentadecanoic acid was crystallized from petroleum (b. p. 40°—60° C) [3].

The B′-form of the same acid was crystallized from the melt [3].

The crystal structure of both forms have been described by the author [4,5].

The C′-form of the same acid can only exist between 46° C and the melting point [3] and it was crystallized from the melt by lowering the temperature just under the melting point. The crystal structure of the C′-form of *n*-hendecanoic acid is being investigated at present by the author.

Table 1. Infrared absorption of normal fatty acids near 5.9 μ (1 700 cm^{-1}), 7.0 μ (1 400 cm^{-1}) and 11.0 μ (900 cm^{-1}). Upper values wavelength (μ), lower values wavenumber (cm^{-1}). (sh) = shoulder.

Fig.	Number of carbon atoms in the acid	Crystal form	Near 5.9 μ (1 700 cm^{-1})		Near 7.0 μ (1 400 cm^{-1})	Near 11.0 μ (900 cm^{-1})	
			Maximum absorption	"Mean value" (see text)		Maximum absorption	Other values
1	15	A′	5.840 1 712	5.860 1 706	7.075 1 413	10.745 931	11.235 11.71 (sh) 890 854
2	15	B′	5.840 1 712	5.865 1 705	7.090 1 410	10.780 928	10.42 (sh) 10.96 (sh) 960 912 11.20 (sh) 11.40 (sh) 893 877
3	15	C′	5.845 1 711	5.860 1 706	7.090 1 410	10.720 933	11.20 (sh) 893
4	15	melt	5.850 1 709	5.850 1 709	7.095 1 409	10.765 929	
5	16	A	5.885 1 699	5.890 1 698	7.100 1 408	10.695 935	10.44 (sh) 10.98 (sh) 958 911 11.260 888
6	16	C	5.840 1 712	5.880 1 701	7.080 1 412	10.650 939	10.50 (sh) 11.235 952 890 11.770 850
7	18	B	5.845 1 711 5.910 1 692	5.880 1 701	7.130 1 403	11.275 887	10.63 (sh) 10.950 941 913 11.440 11.775 874 849
8	18	C	5.865 1 705	5.880 1 701	7.070 1 414	10.625 941	10.50 (sh) 11.225 952 891 11.77 (sh) 850
9	16	melt	5.860 1 706	5.850 1 709	7.080 1 412	10.690 935	

It was not possible to prepare pure A-, B- and C-forms of the same acid with an even number of carbon atoms. Thus two acids were used and the polymorphs were prepared in the following way.

The A-form of palmitic acid was crystallized from petroleum (b. p. 40°—60° C) [3]. The same crystal form of lauric acid is being investigated at present using single crystal X-ray methods.

The B-form of stearic acid was crystallized from petroleum (b.p. 40°—60° C) [7]. Its crystal structure has been previously described [7].

The C-forms of palmitic and stearic acid were crystallized from the melt [3]. The C-form of lauric acid has been described by Vand, Morley and Lomer [8].

Table 2. Infrared absorption of normal fatty acids between 7.7 and 8.5 μ (1 300 and 1 180 cm⁻¹). Upper values wavelength (μ), lower values wavenumber (cm⁻¹).

Fig.	Number of carbon atoms in the acid	Crystal form	Band progression	Near 7.81 μ (1 280 cm⁻¹)	Near 8.07 μ (1 239 cm⁻¹)
1	15	A′	7.715 7.840 7.970 8.120 8.280 8.430 1 296 1 276 1 254 1 232 1 208 1 186		
2	15	B′	7.705 7.830 7.980 8.130 8.285 8.410 1 298 1 277 1 253 1 230 1 207 1 189		
3	15	C′		7.810 1 280	8.065 1 240
4	15	melt		7.800 1 282	8.060 1 241
5	16	A	7.720 7.865 7.985 8.130 8.270 8.355 1 295 1 271 1 252 1 230 1 209 1 197		
6	16	C	7.730 7.870 8.000 8.155 8.295 8.425 1 294 1 271 1 250 1 226 1 206 1 187		
7	18	B	7.750 7.835 7.910 8.010 8.130 8.270 1 290 1 276 1 264 1 248 1 230 1 209		
8	18	C	7.685 7.800 7.915 8.045 8.175 8.310 8.420 1 301 1 282 1 263 1 243 1 223 1 203 1 188		
9	16	melt		7.810 1 280	8.090 1 236

EXPERIMENTAL

A Perkin Elmer model 21 spectrophotometer with sodium chloride prism was used for the infrared absorption measurements. The specimens were investigated as nujol mulls. The temperature in the sample holder was 36° C without extra heating. In the cases where higher temperatures were necessary the sample holder was equipped with a thermometer and isolated with asbestos thread and heated with a heating lamp to the right temperature. All absorption curves, which are linear in wavelength, were calibrated with a polystyrene spectrum and the values are exact up to ± 0.01 μ.

RESULTS AND DISCUSSION

All polymorphs mentioned above as well as liquid *n*-pentadecanoic acid and *n*-palmitic acid were investigated. The absorption curves can be seen in Figs. 1—9 and the wavelengths measured in Table 1 and 2.

The following parts of the spectra are of special interest and will be discussed one by one: near 5.9 μ (1 700 cm⁻¹), near 7.0 μ (1 400 cm⁻¹), band progression 7.7—8.5 μ (1 300—1 180 cm⁻¹) and near 11.0 μ (900 cm⁻¹).

Near 5.9 μ (1 700 cm^{-1})

There is a strong absorption peak near 5.9 μ (1 700 cm^{-1}) which has been attributed to the C=O stretching vibration in dimers. Davies and Sutherland [9] point out that this peak and that belonging to the probable C=O stretching vibration at 7.81 μ (1 280 cm^{-1}) should be double, owing to the dimerization resulting from hydrogen bonding of two carboxylic groups.

In the case of the B-form of stearic acid the peak is clearly resolved into two peaks at 5.845 μ (1 711 cm^{-1}) and 5.910 μ (1 692 cm^{-1}) of the same heights (Fig. 7 and Table 1) and in some of the other cases shoulders can be seen indicating more than one absorption peak. The occurrence of this double peak might also indicate the presence of two different C=O bonds in the crystals of this polymorph but this is not the case [7], so this possibility can be ruled out.

In order to be able to compare the wavelengths with previous results use has been made of a "mean value" which is the wavelength that cuts the whole area of the absorption peak in two equal parts. These "mean values" and the values for maximum absorption are found in Table 1. The "mean values" agree very well with those measured by Sinclair *et al.*[1] and others. It can be seen that all the values for maximum absorption and all the "mean values" lie on or between the two values found for the crystalline B-form of stearic acid. There may possibly be two peaks at these wavelengths in all cases but as their relative intensities may be different the values for maximum absorption and "mean values" may vary between the extreme values.

Near 7.0 μ (1 400 cm^{-1})

There is an absorption peak between 7.07 μ and 7.13 μ (1 414 cm^{-1} and 1 403 cm^{-1}) (Table 1) which has been associated with the deformation of the first CH_2 group which is influenced by the carboxylic group [2,p.147].

In Fig. 3 in the paper by Sinclair *et al.*[1] it can be seen that the position of this peak depends also on the chain-length. As both chain-length and crystal form can vary for the fatty acids this absorption peak alone cannot be used for identification.

Band progression 7.7—8.5 μ (1 300—1 180 cm^{-1})

Jones *et al.*[10] point out that the number and positions of the evenly spaced absorption peaks in this interval are dependent on the chain-length. These absorption wavelengths have been associated with twisting and wagging motions of the methylene groups in the hydrocarbon chains.

In Table 2 it can be seen, that the positions of the peaks are also dependent on the crystal form. The wavelength values for the B-form of stearic acid compared with the C-form of the same acid are higher at lower wavelengths and lower at higher wavelengths in this interval. A comparison between the A- and C-forms of palmitic acid reveals that at lower wavelengths the absorption peaks have the same positions, but that at higher wavelengths the values belonging to the A-form are shifted to shorter wavelengths.

The A′- and B′-forms of *n*-pentadecanoic acid do not show any difference in this region.

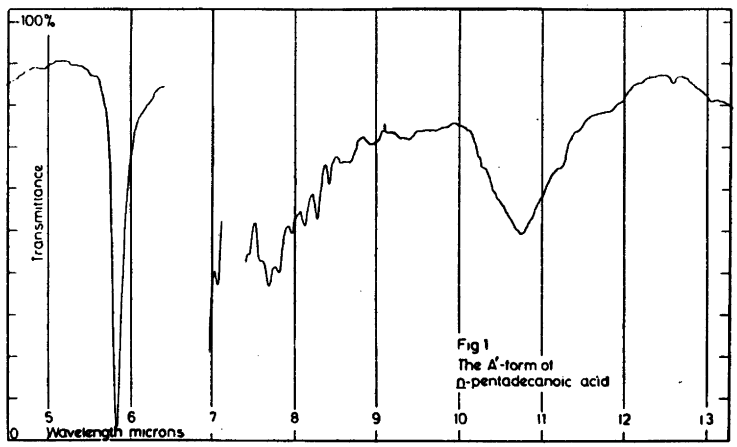

Fig 1
The A'-form of
n-pentadecanoic acid

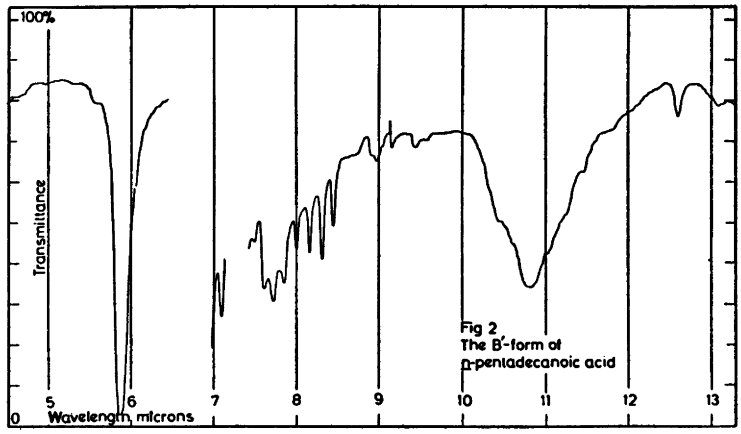

Fig 2
The B'-form of
n-pentadecanoic acid

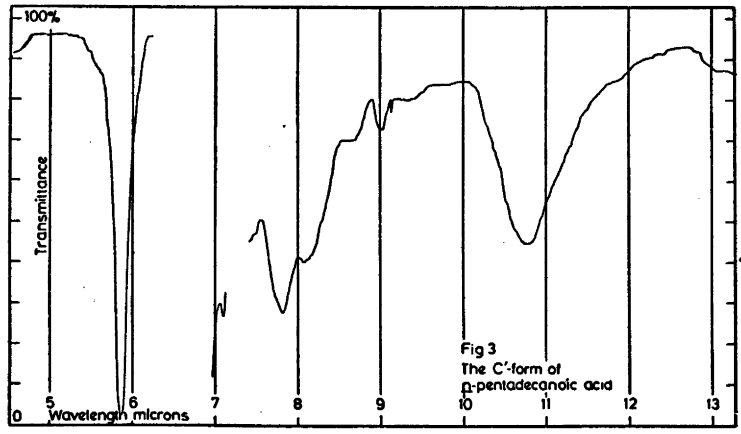

Fig 3
The C'-form of
n-pentadecanoic acid

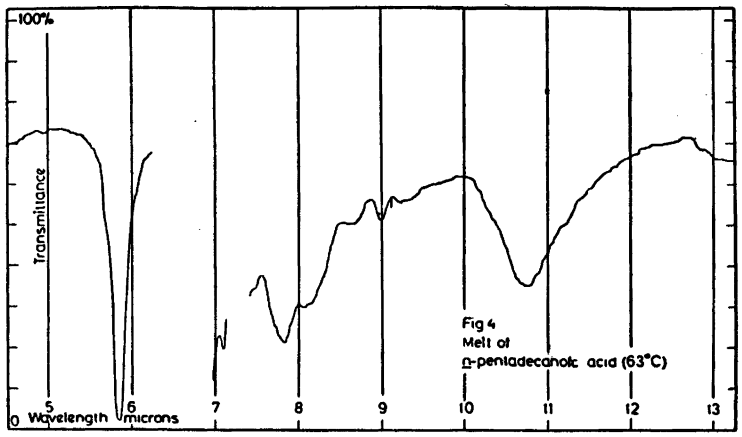

Fig 4
Melt of
Ω-pentadecanoic acid (63°C)

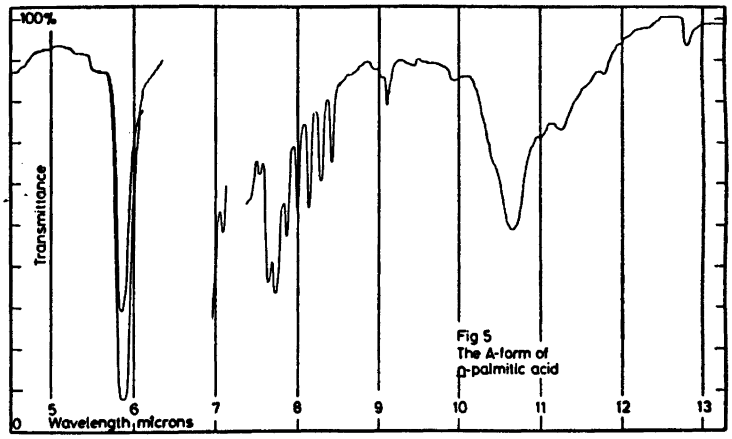

Fig 5
The A-form of
Ω-palmitic acid

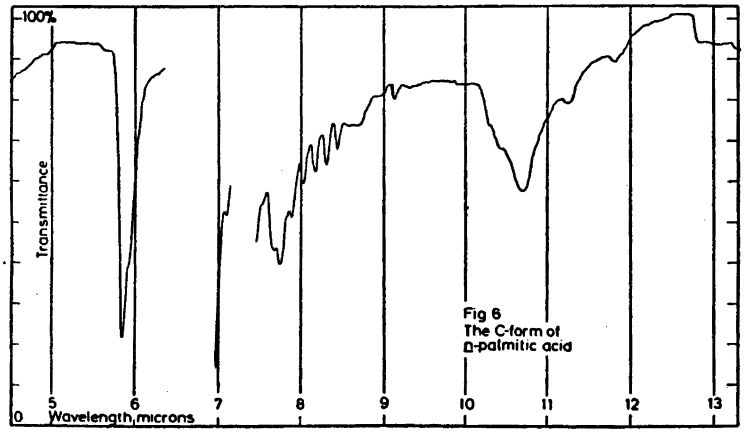

Fig 6
The C-form of
Ω-palmitic acid

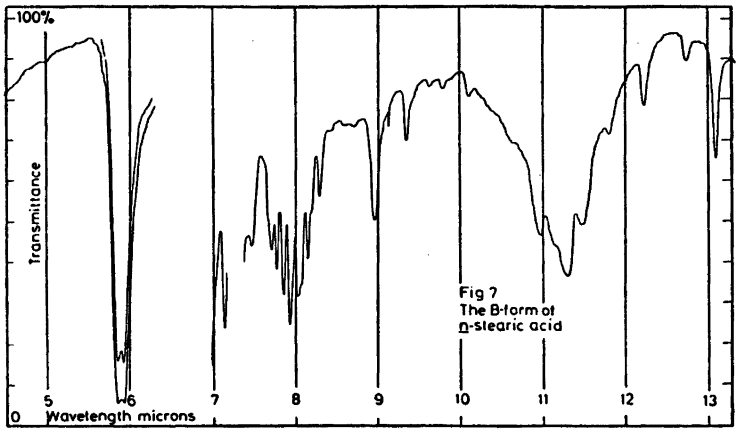

Fig 7
The B-form of
n-stearic acid

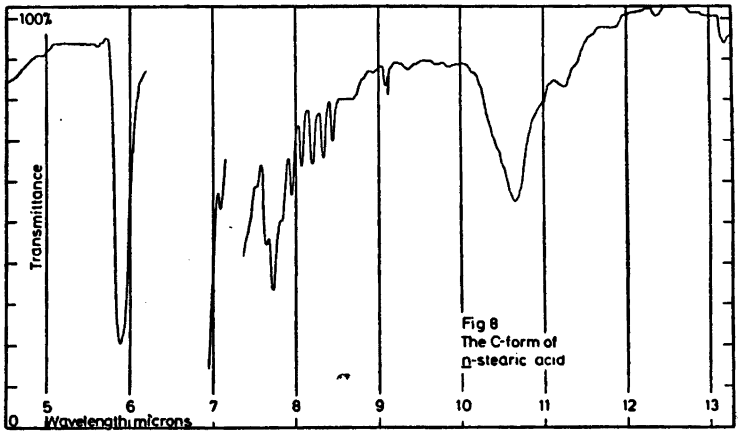

Fig 8
The C-form of
n-stearic acid

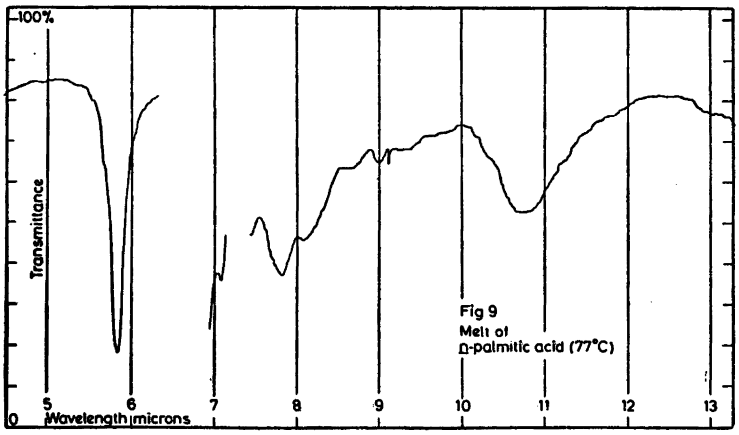

Fig 9
Melt of
n-palmitic acid (77°C)

The C′-form and the melt of *n*-pentadecanoic acid as well as the melt of palmitic acid do not show this band progression. Bellamy [2,p.149] says, however, that even in the liquid state normal fatty acids exhibit this band progression. In a private communication he says that the melted films examined had been re-solidified thus having some degree of orientation.

I think it is rather probable that when the thermal motion increases, the twisting and wagging motions become less regular and there will be no sharp absorption at special wavelengths near and over the melting point.

The spectra of the C′-form and the melt of *n*-pentadecanoic acid as well as the melt of palmitic acid have, however, two peaks at 7.81 μ (1 280 cm^{-1}) and 8.07 μ (1 239 cm^{-1}). Their origins are very uncertain but they certainly have something to do with the carboxylic group.

Near 11.0 μ (900 cm^{-1})

There is an absorption region near 11.0 μ (900 cm^{-1}) which has been associated with the OH out-of-plane deformation [11].

The values for the different crystal forms and melts are tabulated in Table 1. The absorption region consists either of a broad peak with several shoulders and smaller peaks or of a couple of more narrow peaks. The exact origin of all these peaks is very uncertain. However, as no two crystal forms have the same absorption this part of the infra-red spectra is very suitable for identification, particularly in combination with studies of the band progression between 7.7 and 8.5 μ (1 300 and 1 180 cm^{-1}).

In the work by Sinclair *et al.*[1], Fig 3, it can be seen that the value for maximum absorption near 11.0 μ (900 cm^{-1}) is constant for the same crystal form of acids with 14, 16, 18 and 20 carbon atoms (the C-form) and of acids with 17, 19 and 21 carbon atoms (the B′-form), respectively.

The melted acids have very broad absorption peaks without any shoulders or extra peaks.

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On the Reaction between Urea and Formaldehyde in Neutral and Alkaline Solutions

I. Experimental Studies of the Rates of the Equimolecular Reaction

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Some methods for formaldehyde determinations in urea-formaldehyde reaction solutions are examined. A number of effects which have to be considered when applying polarography to this subject are analysed and a suitable supporting electrolyte composition given.

The results of reaction rate studies at 20° C of the equimolecular reaction between urea and formaldehyde at comparatively high concentrations of the reactants are given. From these studies it is found that at a urea-formaldehyde ratio of 1:1 no theoretically significant reaction order is present. The previously reported rapid initial reaction was not observed. In buffered solutions, as in unbuffered, the reaction order was approximately 1.6. In buffered solutions a reaction of second order is present if an excess of urea is used. From such reaction studies rate constants can be calculated. The reaction was found to be subject to acid-base catalysis, and different catalysts show different catalysing power. A linear relationship was found between the rate constants and the amounts of catalyst present. The hydroxyl ion catalysis is not found to be a simple function of the hydroxyl ion activity.

The rates of the reaction

$$H_2N—CO—NH_2 + HCHO = H_2N—CO—NH—CH_2OH \qquad (A)$$

were previously studied by Smythe, Crowe and Lynch, Bettelheim and Cedvall and De'Jong.

By means of a hydroxylamine titration method Smythe [1] investigated the rate of the equimolecular urea-formaldehyde reaction in concentrated, unbuffered solutions at a pH of about 7 and at different temperatures. The reaction product was found to be monomethylol urea, and no dimethylol urea was present in sufficient quantity to be detected. The reaction was of the bimolecular type, except for a rapid initial stage. An activation energy of 14.6 kcal/mole was obtained. Later on, Smythe [2] took a special interest in the

initial rapid reaction. It was found that influence of urea hydrolysis products could not explain this reaction, and repeated experiments, also here made by means of the hydroxylamine method, were in agreement with his earlier observations. In the temperature range 20 – 40° C the initial reaction during the first 300 seconds included 36 – 46 % of the complete condensation reaction. The mechanism of the rapid reaction was also discussed by Smythe [3], and in the same work the effect of small amounts of some compounds present in the reaction solution was also studied. (Na_2HPO_4, $H_2C-COONa$, $H_2N-CH_2-COONa$, $Na_2B_4O_7$, CH_3OH and hydroquinone.) Smythe found that the highest rate was obtained in 1:300 Na_2HPO_4. In this investigation, as in the previous, a change in pH during the initial reaction was observed. Finally, Smythe [4] studied the urea-formaldehyde reaction in dilute solutions by means of polarography. In this case no rapid initial reaction could be found. Similar studies of dilute solutions, also made by means of polarography, are due to Crowe and Lynch [5,6], and the experiments included buffered, alkaline solutions. (0.1 M $NaHCO_3$, pH 8.7; 0.05 M H_3BO_3 + 0.05 M LiCl + 0.045 M LiOH, pH 10.1; 0.1 M Na_2CO_3, pH 11.2; 0.05 M LiOH, pH 12.7.) The reaction was found to be of second order, and the rate was increasing with pH and temperature. An activation energy of 15.9 kcal/mole was calculated.

Bettelheim and Cedvall [7] investigated the urea-formaldehyde reaction in unbuffered, concentrated solutions at 40° C in the neutral and alkaline range. The change in formaldehyde concentration of the reaction mixture was determined by means of a sodium sulfite titration method (Lemme-Doby). Also in this case a bimolecular reaction was found, but the initial reaction was more moderate, only including about 15 % of the complete reaction.

Finally, De'Jong [8] checked the initial rapid reaction in solutions prepared from repeatedly crystallised urea, and in this investigation no reaction of such a kind could be found. However, in the experiments an excess of urea was used. The previously observed initial reaction was assumed to be due to ammonia impurities, and these impurities would react with the formaldehyde rapidly. De'Jong did not mention what formaldehyde analysing method he used.

The purpose of the following paper is to examine the methods for formaldehyde determination in urea-formaldehyde reaction mixtures and to describe experimental studies at 20° C with special regard to the reaction order and the influence of pH and buffer substances.

DETERMINATION OF FORMALDEHYDE IN UREA–FORMALDEHYDE REACTION SOLUTIONS

The use of *polarography* for determination of formaldehyde in urea-formaldehyde reaction solutions was primarily introduced by Crowe and Lynch [5,6]. From formaldehyde limiting current measurements in solutions containing the same amount of buffer substances and of the same pH as the reaction mixtures, the formaldehyde was determined directly in these mixtures. Smythe [4] used a somewhat different method: Samples from the reaction solutions were analysed on formaldehyde polarographically at 25° C; the supporting electrolyte was 0.095 M Na_2HPO_4 + 0.022 M KH_2PO_4, pH 7.15. Calibration curves gave a linear relationship between limiting current and formaldehyde concentration, except for small deviations at low formaldehyde concentrations. These deviations are probably due to difficulties when evaluating the polarograms; this seems to be probable since Na^+ and K^+ are the cations of the supporting electrolyte and the waves of these ions interfere with the formaldehyde wave. The evalution difficulties are also increased because the formaldehyde current-voltage curve shows a maximum at high concentrations (Smythe [4], p. 575, Fig. 2). Smythe mentions that methylol urea acts as a maximum suppressor,

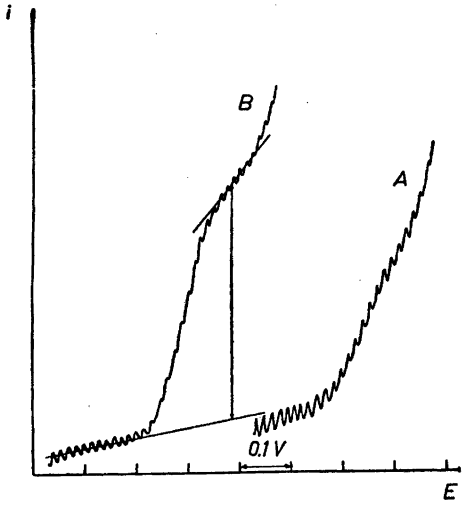

Fig. 1. Formaldehyde polarograms of a urea — formaldehyde reaction solution, $C_{Na^+} = 0.2$; obtained by means of Smythe's method (A) and the method described in this paper (B).

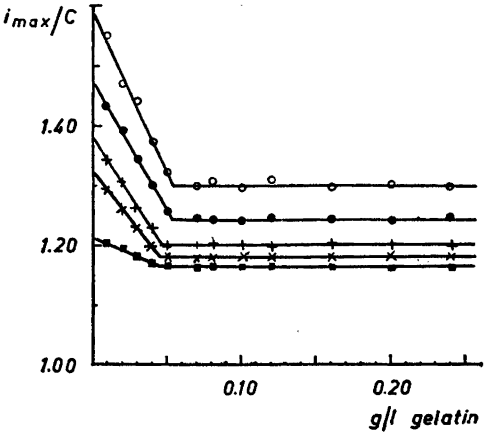

Fig. 2. The maximum suppression effect of gelatin at different formaldehyde concentrations: 0.150 (○), 0.100 (●), 0.060 (+), 0.025 (×) and 0.010 (■) at 25° C. Supporting electrolyte: 0.10 M Li_2CO_3, pH 9.50.

but this effect is of course not present at the beginning of the urea-formaldehyde reaction. Within the limits of the experimental conditions used by Smythe, the method seems to be applicable. However, the determination becomes more complicated if the reaction mixtures contain a considerable amount of Na^+ due to buffer substances present. When the sodium and formaldehyde waves interfere, the accuracy is often low. An example is given in Fig. 1, polarogram A, representing a 15 % urea-formaldehyde reaction mixture, the molar ratio of urea to formaldehyde is 1:1.5 and 6 % formaldehyde is still unreacted. The Na^+ concentration is 0.2 M, and the polarogram was recorded at 25° C with a supporting electrolyte according to Smythe and by means of a LKB type 3 266 polarograph. The dilution ratio was 1:20. Air oxygen was present and no maximum suppressor used. The drop time of the mercury electrode was 3.5 sec. From the diagram it is found that we have reasons to look for a supporting electrolyte which is more generally applicable.

Since the cation of the supporting electrolyte ought to have as negative half wave potential as possible, in order to reduce the interference, $(CH_3)_4N^+$ would be suitable. However, this ion might cause some uncontrolled reactions in solutions containing methylol urea and formaldehyde, and therefore it might be better to use Li^+. The half wave potential of Li^+ is more negative than that of Na^+, the difference being 0.16 V, and this would decrease the difficulties mentioned above, especially if a suitable pH of the supporting electrolyte is chosen. As shown by Bieber and Trümpler [9], the half wave

potential of formaldehyde is dependent on pH; increasing pH gives a more negative half wave potential. This effect, which is undesirable from the point of view of interference, is compensated by the increasing rate of methylene glycol dehydration, *i. e.* the ratio between limiting current and formaldehyde concentration increases with increasing pH; Bieber and Trümpler [9]. A number of preliminary experiments carried out by the present author, gave the result that the higher the pH, the easier the evaluation of the polarograms.

Methylol urea is hydrolysed in alkaline solutions, which was shown by Crowe and Lynch [5], and this effect has to be regarded. The results of another set of preliminary experiments made by the present author were that the rate of hydrolysis is dependent on:

1. pH; the rate of hydrolysis increasing with increasing pH.
2. Temperature; the rate of hydrolysis increasing with increasing temperature.
3. Dilution of the reaction mixture with supporting electrolyte; the rate of hydrolysis increasing with increasing dilution.

(Some of these results were previously known from Crowe and Lynch [5])

Of course, the reaction between urea and formaldehyde can also continue in the polarographic solution, and further preliminary experiments gave the results that the rate of this reaction is dependent on 1 and 2 in the same way as the hydrolysis. The opposite effect is present in the case 3, decreasing reaction rates were found when the dilution was increased.

The influence of the buffer concentration of the supporting electrolyte on the reactions mentioned above was not studied, because the concentration is limited by the solubility of the Li^+ salts, and because a concentration close to this limit has to be used. This is necessary since otherwise the hydroxyl ions produced at the electrode surface during the reduction of formaldehyde

$$HCHO + 2\ H_2O = CH_3OH + 2\ OH^- \qquad (B)$$

will change the acid-base catalysis of the electrode reaction, *i. e.* will reduce the linear interval of the current concentration curve, as can be concluded from Landqvist [10]. (For further informations concerning the theory of formaldehyde polarography, reference is given to that paper.)

When considering the interference, the hydrolysis and the continuing reaction mentioned above, 0.1 M Li_2CO_3, pH 9.5 might be a suitable supporting electrolyte. The solubility of Li_2CO_3 does not permit a higher concentration than 0.1 M, and no other suitable buffer substance for the pH range in question could be found. The pK'' of the carbonate at the ionic strength here present is of the order 9.8, and this means that a slight influence of of hydroxyl ions at moderate and high limiting currents may be expected.

As regards the temperature of the polarographic solutions, an increased temperature is likely to cause an increased limiting current-formaldehyde concentration ratio, *i. e.* gives a favourable effect. However, at the same time the increased rate of the reactions previously mentioned is unfavourable. Because the temperature coefficient of the limiting current is high (Crowe and Lynch [6], Fig. 1), it is necessary to maintain a good temperature constancy. From the point of view of thermostating a temperature a little above the room temperature is useful, *i. e.* 25° C is in most cases to be preferred.

As mentioned before, the polarographic current-voltage curve of formaldehyde shows a maximum. In very dilute solutions this effect is not present, but increasing concentration will cause more pronounced maxima. Some preliminary experiments made by the present author gave the information that maxima are pH-dependent, at pH 6.9 and 25° C no maxima were present when $C_{(HCHO)a}$, *i. e.* the analytical formaldehyde concentration, was below 0.05 M. However, at pH 9.50 and the same concentration and temperature, this effect cannot be neglected. The increased tendency to maxima could be regarded as a function of the magnitude of the limiting current, but in still more alkaline solutions the effect decreases. In 0.05 M LiOH, for instance, no maxima can be found at the concentration and temperature mentioned above.

Since from other points of view a supporting electrolyte pH of 9.5 is desirable, we need to reduce the tendency to form maxima, *i. e.* to find a maximum suppressor. The only one of the generally known suppressors which gives a sufficient effect even at low concentrations of the substance, seems to be gelatin. Unfortunately gelatin is not a chemically defined product, and it is probably wise to be careful when changing from one gelatin to another. When expecting a large "consumption" of the substance, *e. g.* for a research project or for industrial control purposes, a comparatively large amount can be kept in stock, so that the same product is available for the whole investigation. The gelatin is preferably dried and grinded dry. After grinding the gelatin is thoroughly mixed. Before using a batch it might be tested to find the appropriate concentration at the highest amounts of formaldehyde expected.

In the following an investigation is described the purpose of which was to study the relationship between maximum suppression and formaldehyde and gelatin concentrations. The experiments were made in an open cell, *i. e.* dissolved oxygen was present, and at 25° C. The drop time of the mercury electrode was 3.4 sec. and the mercury flow 1.57 mg/sec. The supporting electrolyte was 0.10 M Li_2CO_3, pH 9.50. The investigation included 5 formaldehyde concentrations, covering the range 0.00—0.15 M. All chemicals were of A. R. quality, except for formaldehyde, which was a Merck product of high purity, only containing traces of methyl alcohol and formic acid.* The reference electrode was of the saturated calomel type.

In Fig. 2 the function $I_{max}/C_{(HCHO)a}$, where I_{max} = maximum current and $C_{(HCHO)a}$ = analytical formaldehyde concentration, is plotted as a function of the gelatin concentration in g/l. From this study we find the following:

1. Maxima are dependent on the formaldehyde concentration; increasing concentrations give rise to increased maxima.
2. A limiting gelatin concentration is present, *i. e.* at a concentration above this limit no maxima occur.
3. The limiting concentration is dependent on the amount of formaldehyde present, but to a comparatively low degree.

The findings 2 and 3 are in accordance with the idea that one kind of polarographic maximum suppression mechanism includes the adsorption of the maximum suppressor at the electrode surface (Heyrovsky [11]).

* $< 2 \cdot 10^{-4}$ M at 380 g/l formaldehyde.

When gelatin is used in solutions containing formaldehyde, the possibility of reactions between these two components cannot be neglected. In order to find if a reaction occur when applying the conditions mentioned above, $I_{max}/C_{(HCHO)a}$ was measured in the supporting electrolyte in question and at a gelatin concentration of 0.20 g/l and a formaldehyde concentration of 0.15 M. The temperature was 25° C as before. During the 60 minutes studied no time dependence could be observed, *i. e.* no gelatin-formaldehyde reaction was found. It is probably necessary to apply the same test to every new batch of gelatin, since it cannot *a priori* be postulated that the results here obtained are valid for any type of gelatin.

From Fig. 2 it is seen that the limiting concentration of gelatin is of such an order that 0.12 g/l is sufficient in the here actual concentration range.

Smythe [4] reports that methylol urea acts as a maximum suppressor, however, preliminary experiments made by the present author gave the result that the limiting concentration of this suppressor was comparatively high: 2—3 g/l. Such a high concentration will decrease the accuracy at low formaldehyde concentration determinations too much, since the equilibrium formaldehyde concentration of methylol urea in such a case can exceed the amount to be determined. Thus, the use of monomethylol urea as a maximum suppressor cannot be recommended.

In Fig. 1, polarogram B is obtained by means of a supporting electrolyte comprising 0.10 M Li_2CO_3, pH 9.50, 0.12 g/l gelatin; the temperature was 25° C. The reaction mixture investigated was the same as that of curve A of the same figure. The result will clearly explain that the method is suitable also in more difficult cases.

Fig. 3 shows a calibration curve, *i. e.* the limiting current — formaldehyde concentration relationship; supporting electrolyte as above, drop time 3.4 sec. and flow of mercury 1.62 mg/sec. At high aldehyde concentrations the hydroxyl ion effect is present and the linearity lost. However, the linearity is good up to 0.06 M formaldehyde, but attention must be drawn to the fact that this concentration is not of a general significance, as it relates to the conditions of the capillary here used. This since the "diffusion layer" of hydroxyl ion neutralisation by the supporting electrolyte buffer substances and the corresponding layer of methylene glycol dehydration and formaldehyde reduction are entirely different. Thus, for each capillary the corresponding calibration curve has to be obtained experimentally. Theoretical calculations in this field seem to be very complicated.

For some of the formaldehyde concentrations in Fig. 3, four polarograms were recorded, 3, 6, 12 and 18 minutes from the moment when a 1.0 M formaldehyde stock solution and the supporting electrolyte were mixed. No influence of time could be found, *i. e.* the rate of depolymerisation of any formaldehyde polymers present was in this case sufficiently high to prevent any disturbances.

In order to investigate the role of continuing reaction and hydrolysis in the polarographic solution when the here studied supporting electrolyte is used, the following reactions were investigated by means of polarography: 4.0 M urea + 4.0 M formaldehyde, and 4.0 M urea + 8.0 M formaldehyde. The solutions were 0.1 M with respect to Na_2CO_3 and had a pH of 9.80. The

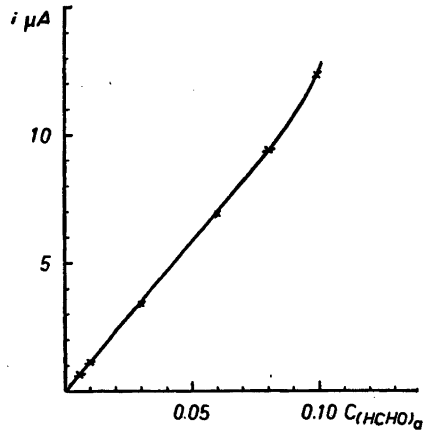

Fig. 3. A limiting current — formaldehyde concentration curve at 25° C. Supporting electrolyte: 0.10 M Li_2CO_3 + 0.12 g/l gelatin, pH 9.50.

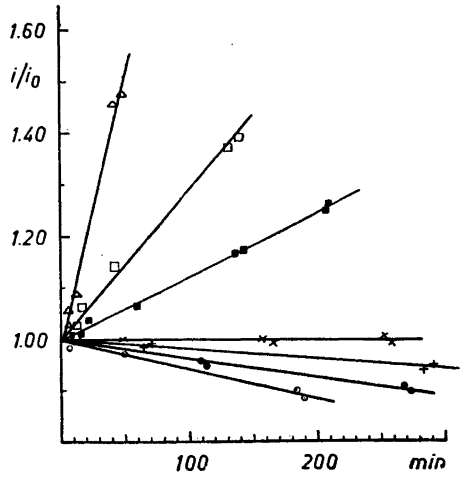

Fig. 4. Continuing reaction and hydrolysis effects in 0.10 M Li_2CO_3 + 0.12 g/l gelatin, pH 9.50. The temperature was 25° C and the dilution 1:100. Reaction solution: 4.0 M $CO(NH_2)_2$ + 4.0 M HCHO in 0.10 M Na_2CO_3, pH 9.80 and at 20° C. The curves are related to the following degree of reaction: 1 % (△), 26 % (□), 33 % (■), 52 % (×), 78 % (+), 86 % (●) and 94 % (○).

reaction temperature was 20° C. The polarography was carried out at 25° C and the dilution ratio was 1:100. These conditions seem to be "unfavourable" as regards the effects to be studied. The results obtained can be found in Figs. 4 and 5, where the ratio between the actual limiting current and the current extrapolated to zero time is plotted against the time from mixing the sample and the supporting electrolyte. For both reactions we find that in the beginning of the urea-formaldehyde reaction this reaction continues in the polarographic solution. However, during the later parts of the reaction, hydrolysis is the most important effect. The curves of the functions are approximately straight lines, and for accurate determinations the zero time extrapolation mentioned above can easily be applied. If the accuracy need is moderate — of the order 3 % — it is sufficient to record the polarograms within 5 minutes from mixing. It was further observed that within the limits of the linear part of the current-concentration curve, no influence of the dilution ratio (1:5—1:100) on the polarographic result was found. However, it must always be regarded that the dilution does not change the buffer concentration of the polarographic solution so much that the calibration curve becomes invalid. The effects here mentioned were also found when a supporting electrolyte in accordance with Smythe was used.

From this it is seen that the use of polarography in studies of the urea-formaldehyde reaction rates requires the consideration of a number of effects.

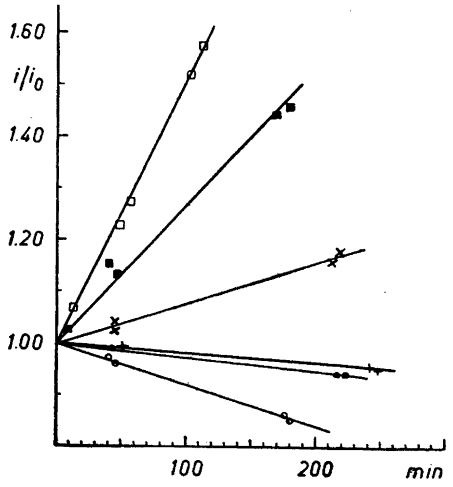

Fig. 5. Continuing reaction and hydrolysis effects in 0.10 M Li_2CO_3 + 0.12 g/l gelatin, pH 9.50. The temperature was 25° C and the dilution 1:100. Reaction solution: 4.0 M $CO(NH_2)_2$ + 8.0 M HCHO in 0.10 M Na_2CO_3, pH 9.80 and at 20° C. The curves are related to the following degree of reaction: 10 % (□), 39 % (■), 70 % (×), 82 % (+) and 86 % (○).

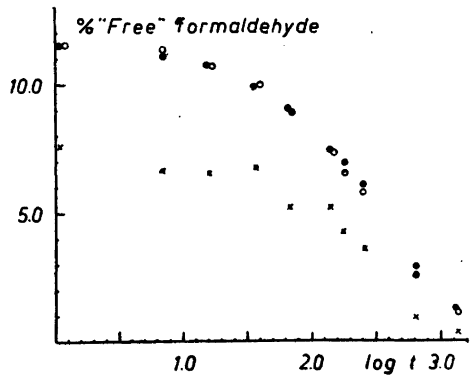

Fig. 6. Reaction between 3.92 M $CO(NH_2)_2$ + 3.92 M HCHO at 20° C in an unbuffered solution with the initial pH 8.0. Per cent "free" formaldehyde of the reaction solution was determined by means of: polarography (●), the sulfite method (○) and the hydroxylamine method (×).

However, when such a consideration is given, the polarographic method offers several advantages, *e. g.* reliability, speed and accuracy.

In order to examine *the titration methods* previously applied (Smythe, Bettelheim and Cedvall) two experiments were carried out, involving the reaction between 3.92 M $CO(NH_2)_2$ + 3.92 M HCHO and 1.96 M $CO(NH_2)_2$ + 3.92 M HCHO at 20° C in unbuffered solutions with an initial pH of 8.0. These reactions were studied by means of: a) the polarographic method described in this paper, b) the hydroxylamine titration method, and c) the sulfite titration method.

The hydroxylamine titration method was applied as follows:

2 ml of the reaction mixture were measured by a pipet into a 100 ml Erlenmeyer flask, 10 ml distilled water added, 10 drops of bromophenol blue indicator, followed by 20 ml 10 % by weight hydroxylamine hydrochloride solution. (This represents 79 % hydroxylamine hydrochloride excess at the highest formaldehyde concentration.) Exactly 30 seconds from the midpoint of the time taken for the pipet to deliver 20 ml solution, the titration of the liberated hydrochloric acid with 0.5 M NaOH begun and was completed within 1—2 minutes. The endpoint was matched against a standard. (Smythe [1].)

The sulfite method was handled in accordance with the following description:

2 ml of the reaction mixture were measured by a pipet into a 100 ml Erlenmeyer flask, 10 ml distilled water and 5 drops of thymolphthalein indicator added, followed by 25 ml 15 % by weight (anhydrous) sodium sulfite solution. (This amount of sodium sulfite

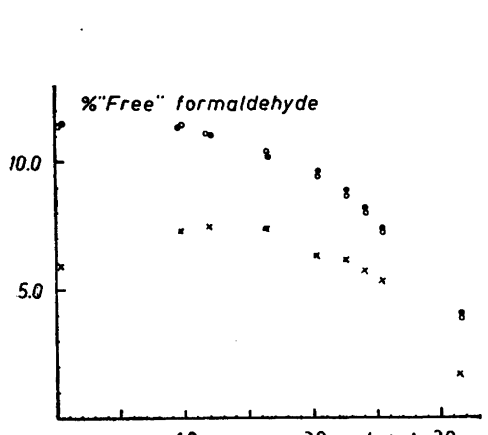

Fig. 7. Reaction between 1.96 M $CO(NH_2)_2$ + 3.92 M HCHO at 20° C in an unbuffered solution with the initial pH 8.0. Per cent "free" formaldehyde of the reaction solution was determined by means of: polarography (●), the sulfite method (○) and the hydroxylamine method (×).

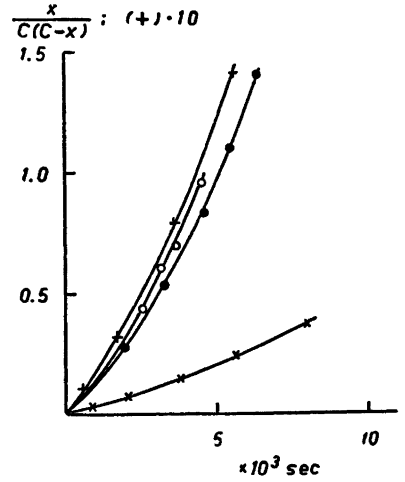

Fig. 8. The reaction between 4.0 M $CO(NH_2)_2$ + 4.0 M HCHO at 20° C in: an unbuffered solution, initial pH 6.70 (×); 0.10 M KH_2PO_4, pH 6.70 (●); 0.10 M borax, pH 9.20 (×) and 0.10 M Na_2CO_3, pH 9.80 (○).

represents 87 % excess at the highest formaldehyde concentration.) The sulfite solution was neutralised against an indicator standard (pale blue), which also was used for the titration with 0.5 M HCl. The titration begun exactly 1 minute from the midpoint of the sulfite delivery.

The results of these comparative investigations are given in Figs. 6 and 7, where the amount of "free" formaldehyde of the reaction solution is plotted against the logarithm of the reaction time in minutes, as determined by means of the different analytical methods. From the results it is found that the agreement between the polarographic and the sulfite method is good. The hydroxylamine method, however, gives different results. The values obtained were generally too low; in the beginning of the reaction a figure about 35 % below the theoretical value was obtained. The influence of the time used for completing the titration, *i. e.* the time from the start of a titration 30 seconds after mixing and to a completed titration, is eludicated by the following experiment. A reaction sample containing 4.20 % "free" formaldehyde (polarography and sulfite method) was analysed by means of the hydroxylamine method as described above, 10 ml sample used, and the titration was completed at different time delays. The results were:

Titration time min.	% »free» formaldehyde obtained
0.5	3.49
1.0	4.12
1.5	4.50
2.0	4.74

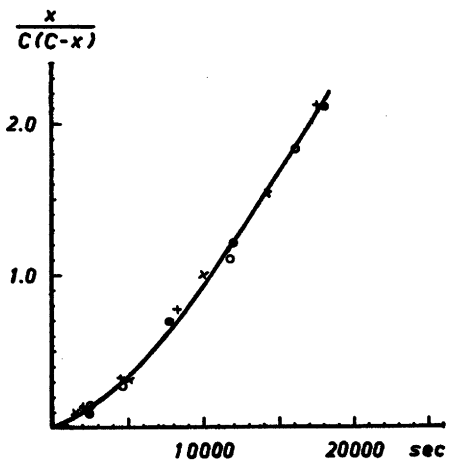

Fig. 9. The reaction in 0.05 M Li_2CO_3, pH 9.50 at 20° C between: 4.0 M $CO(NH_2)_2$ + 4.0 M HCHO (○), 2.0 M $CO(NH_2)_2$ + 2.0 M HCHO (●), 1.0 M $CO(NH_2)_2$ + 1.0 M HCHO (×) and 0.5 M $CO(NH_2)_2$ + 0.5 M HCHO (+).

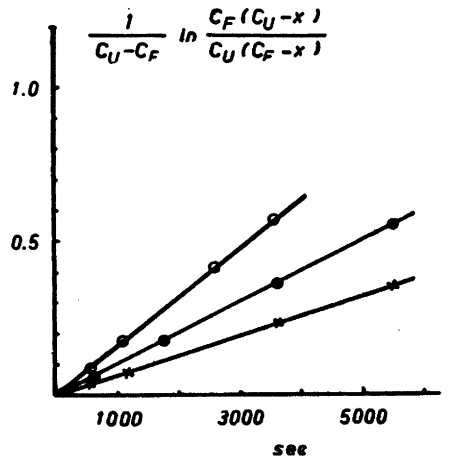

Fig. 10. The reaction between 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO at 20° C, pH 6.70 and $C_{KH_2PO_4}$: 0.1 (○), 0.05 (●) and 0.025 (×).

From this it might be concluded that the time interval "1—2 minutes" is not defined sufficiently sharply when a high accuracy is needed.

The influence of the amount of reaction mixture sample used for the titrations can be found from the following data, obtained by analysing a reaction

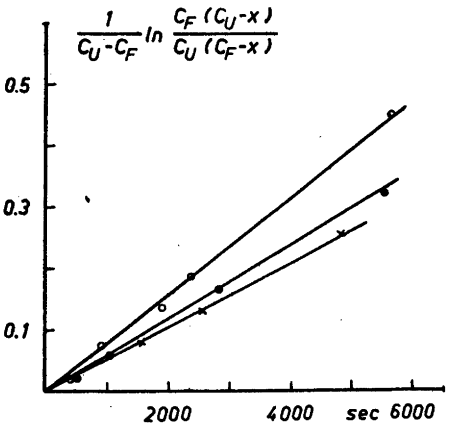

Fig. 11. The reaction between 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO at 20° C, pH 9.20 and C_{borax}: 0.1 (○), 0.05 (●) and 0.025 (×).

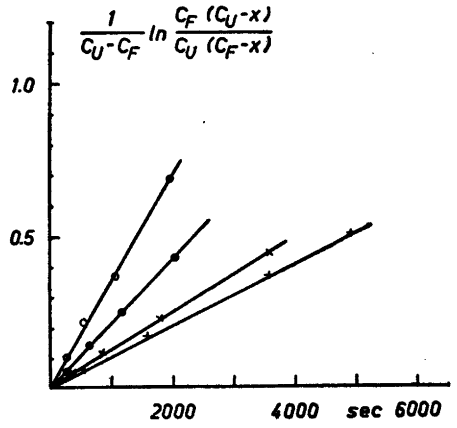

Fig. 12. The reaction between 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO at 20° C, pH 10.00 and $C_{Na_2CO_3}$: 0.2 (○), 0.1 (●), 0.05 (×) and 0.025 (+).

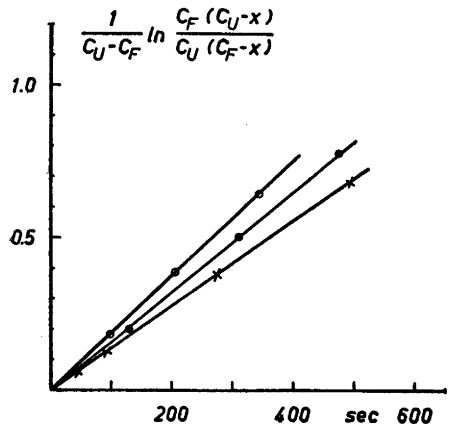

Fig. 13. The reaction between 4.0 M $CO(NH_2)_2$+0.4 M HCHO at 20° C, pH 11.5 and $C_{Na_2CO_3}$: 0.2 (○), 0.1 (●) and 0.05 (×).

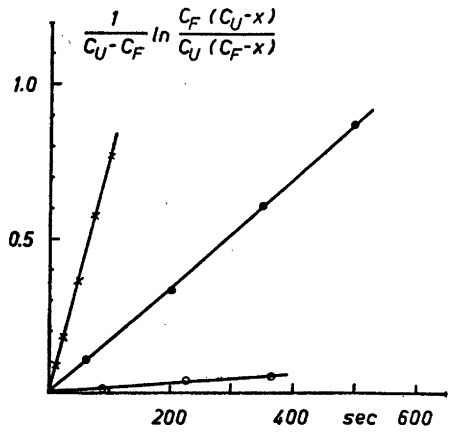

Fig. 14. The reaction between 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO at 20° C and: 0.1 M $Na_4P_2O_7$, pH 8.50 (○); 0.1 M Na_3PO_4, pH 11.50 (●) and 0.1 M Na_3PO_4, pH 12.40 (×).

solution containing 4.30 % "free" formaldehyde by means of the hydroxylamine method, titration time 1.5 minutes: 2 ml sample: 1.65 %, 5 ml sample: 2.97 % and 10 ml sample: 4.27 % "free" formaldehyde.

A similar experiment made by means of the sulfite method gave the results: 2 ml sample: 4.27 % and 5 ml sample: 4.33 % "free" formaldehyde. Within the practical limits of titration times no influence similar to that described for the hydroxylamine method could be found in this case.

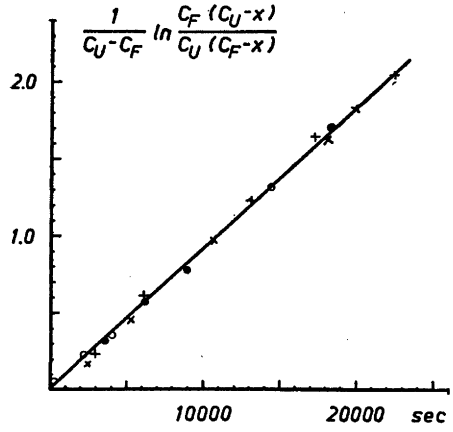

Fig. 15. The reaction in 0.05 M Li_2CO_3, pH 9.50 at 20° C. C_{HCHO} = 0.10 and $C_{CO(NH_2)_2}$: 4.0 (○), 2.0 (●), 1.0 (×) and 0.5 (+).

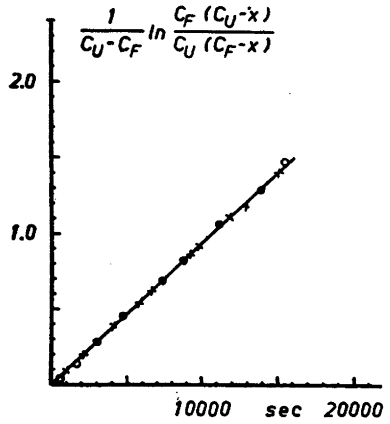

Fig. 16. The reaction in 0.05 M Li_2CO_3, pH 9.50 at 20° C. $C_{CO(NH_2)_2}$ = 4.0 and C_{HCHO}: 0.50 (○), 0.25 (●), 0.10 (×) and 0.05 (+).

It seems to be probable that a highly standardized hydroxylamine method will give a better accuracy than that here obtained. However, in the beginning of the urea-formaldehyde reaction the method might give results which are below the real values also in such a case. From this we find that the hydroxylamine method cannot be recommended.

The sulfite method seems to be more satisfactory; however, both of the titration methods include some disadvantages: After adding the reaction sample the pH of the titration solution will change anomalously, and in some cases this will cause a continuing reaction or hydrolysis before the titration is completed. Of course, this effect can be partly reduced if a buffer substance is added to the solution, *e. g.* a formic acid buffer in the case of the hydroxylamine method and a carbonate buffer in the case of the sulfite method. (These buffers must be preneutralised and the titration stopped at the same pH as used for the preneutralisation.) A more serious disadvantage is that the methods fail when applied to reaction solutions containing buffer substances which have their buffering intervals in a pH range where the titration must be completed. A simple oxidation method, applicable to the sulfite titration method, cannot easily be found, otherwise such a method would solve the problems in question. Another possibility would be the use of ion exchange resins for removing the buffer substances; such a method, however, seems to be comparatively slow.

Thus, when considering a general applicability, the polarographic method might be preferred.

EXPERIMENTAL STUDIES OF THE EQUIMOLECULAR UREA-FORMALDEHYDE REACTION IN NEUTRAL AND ALKALINE SOLUTIONS

The experiments were carried out in a closed flask or an open beaker at 20° C. At this temperature no difference could be found between the results obtained by means of a closed or an open vessel. The reaction mixture was kept at a constant temperature as follows: The vessel was immersed into a water thermostat, kept at 25 ± 0.1° C, and in the vessel a cooling coil was fitted. Through the coil water at 10° C was circulated by a pump. The water circulation was on-off regulated by a contact thermometer in the reaction solution, and this solution was kept in motion by a stirrer. This device gave a temperature constancy within ±0.1° C.

The solutions, containing the amount of buffer substances to be used, were made up to twice the reaction concentration of urea and formaldehyde. All the chemicals were of A. R. quality, except for the formaldehyde, which was a Merck product of high purity, only containing traces of methyl alcohol and formic acid.* The pH of the solutions was then adjusted by hydrochloric acid or the hydroxide of the buffer cation; for the pH measurements the glass electrode technique was used, and at high pH values the differential method in accordance with Landqvist [12] was applied. The accuracy was ±0.02 pH. The "buffer substances" mentioned above also included a chloride of the cation, added in such an amount that the cationic strength in all cases was the same as at the highest buffer concentration. This was made in order to keep the concentration-activity ratio for the buffer anions at different amounts of buffer as constant as possible. The reaction solutions were comparatively concentrated as regards the reactants, since such solutions are of greatest practical interest. In each experiment samples were taken from the reaction mixture at different time delays from the mixing of the reactant solutions, the pH constancy checked (no differences found) and the "free" formaldehyde content determined polarographically by means of the method previously described.

* $< 2.10^{-4}$ M at 350 g/l formaldehyde.

One set of experiments was made with solutions containing urea and formaldehyde in the molecular ratio 1:1. In this case we have the following reaction rate equation, if we assume that monomethylol urea is the reaction product and neglect the hydrolysis reaction:

$$\frac{dt}{dx} = k\ (C-x)^2 \qquad t = 0,\ x = 0$$

and

$$\frac{x}{C\ (C-x)} = k \cdot t \tag{1}$$

where x = monomethylol urea concentration, C = initial urea and formaldehyde concentration, k = reaction rate constant and t = time of reaction.

Another set of experiments were carried out on solutions containing an excess of urea, and in this case we have:

$$\frac{dx}{dt} = k\ (C_U-x) \cdot (C_F-x)$$

where C_U = initial urea concentration and C_F = initial formaldehyde concentration.

From this we obtain:

$$\frac{1}{C_U-C_F} \cdot \ln \frac{C_F\ (C_U-x)}{C_U\ (C_F-x)} = k \cdot t \tag{2}$$

or, when $C_F \ll C_U$:

$$\frac{1}{C_U} \cdot \ln \frac{C_F}{C_F-x} = k \cdot t \tag{2a}$$

The experimental conditions and results can be found in Figs. 8—16, and they are summarized in Table 1.

In all experiments a small volume contraction occurred, of the order 1 %, and correction has been applied for this effect.

In the unbuffered solution at pH 6.70 a small change in pH occurred during the reaction. However, the same curve was obtained when the pH was kept constant by adding NaOH.

From the second order reaction relationship found in solutions containing an excess of urea, reaction rate constants can be calculated. If we plot these constants for each buffer substance at a constant pH as functions of the buffer concentrations, we get a relationship as shown by Fig. 17. The linearity of the curves is good, *i. e.* the concentration-activity ratio is fairly constant, as can be expected from the constant cationic strength of the solutions. The different catalytic properties of the different buffer substances clearly appear from the diagram. If the curves of Fig. 17 are extrapolated to zero buffer concentration, the sum of the "zero reaction rate" and the hydroxyl ion catalytic contribution, k_0, can be calculated. The relation between the logarithm of k_0 and pH is shown in Fig. 18.

Table 1. Experimental data of the equimolecular urea-formaldehyde reaction in neutral and alkaline solutions at 20° C.

Fig.	$C_{CO(NH_2)_2}$	C_{HCHO}	Buffer substance	C_{buffer}	pH	Reaction order	$k \cdot 10^3$
8	4.00	4.00	—	—	6.70 *	—	—
8	4.00	4.00	KH_2PO_4	0.100	6.70	—	—
8	4.00	4.00	borax	0.100	9.20	—	—
8	4.00	4.00	Na_2CO_3	0.100	9.80	—	—
9	4.00	4.00	Li_2CO_3	0.050	9.50	—	—
9	2.00	2.00	Li_2CO_3	0.050	9.50	—	—
9	1.00	1.00	Li_2CO_3	0.050	9.50	—	—
9	0.50	0.50	Li_2CO_3	0.050	9.50	—	—
10	4.00	0.40	KH_2PO_4	0.100	6.70	2	0.16
10	4.00	0.40	KH_2PO_4	0.050	6.70	2	0.10
10	4.00	0.40	KH_2PO_4	0.025	6.70	2	0.065
11	4.00	0.40	borax	0.100	9.20	2	0.080
11	4.00	0.40	borax	0.050	9.20	2	0.060
11	4.00	0.40	borax	0.025	9.20	2	0.052
12	4.00	0.40	Na_2CO_3	0.200	10.00	2	0.34
12	4.00	0.40	Na_2CO_3	0.100	10.00	2	0.20
12	4.00	0.40	Na_2CO_3	0.050	10.00	2	0.12
12	4.00	0.40	Na_2CO_3	0.025	10.00	2	0.10
13	4.00	0.40	Na_2CO_3	0.200	11.50	2	1.9
13	4.00	0.40	Na_2CO_3	0.100	11.50	2	1.6
13	4.00	0.40	Na_2CO_3	0.050	11.50	2	1.4
14	4.00	0.40	$Na_4P_2O_7$	0.100	8.50	2	0.13
14	4.00	0.40	Na_3PO_4	0.100	11.50	2	1.6
14	4.00	0.40	Na_3PO_4	0.100	12.40	2	7.5
15	4.00	0.10	Li_2CO_3	0.050	9.50	2	0.091
15	2.00	0.10	Li_2CO_3	0.050	9.50	2	0.091
15	1.00	0.10	Li_2CO_3	0.050	9.50	2	0.091
15	0.50	0.10	Li_2CO_3	0.050	9.50	2	0.091
16	4.00	0.50	Li_2CO_3	0.050	9.50	2	0.091
16	4.00	0.25	Li_2CO_3	0.050	9.50	2	0.091
16	4.00	0.10	Li_2CO_3	0.050	9.50	2	0.091
16	4.00	0.05	Li_2CO_3	0.050	9.50	2	0.091

* initial pH; final pH was 6.50.

From these experiments the following conclusions might be drawn:

1. The polarographic and the sulfite method for formaldehyde determinations in urea-formaldehyde reaction solutions give similar results (Figs. 6 and 7).

2. The hydroxylamine method gives, especially in the beginning of the urea-formaldehyde reaction, too low formaldehyde concentration values (Figs. 6 and 7).

3. The accuracy of the hydroxylamine method seems to be moderate (Figs. 6 and 7).

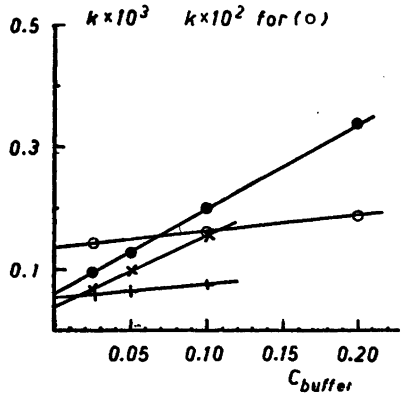

Fig. 17. Relationsship between the reaction rate constant of 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO at 20° C and the buffer substance concentrations: Na_2CO_3, pH 11.5 (○), Na_2CO_3, pH 10.0 (●), borax, pH 9.2 (+) and KH_2PO_4, pH 6.7 (×).

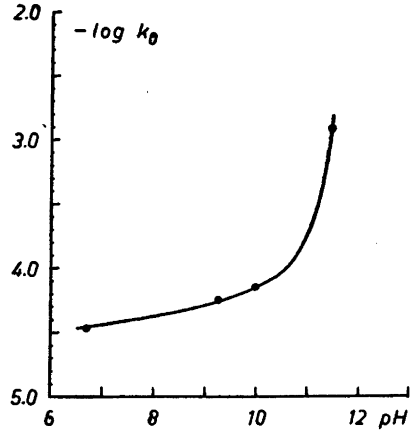

Fig. 18. Relationship between the logarithm of the reaction rate constant at zero buffer concentration and pH for the reaction 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO at 20° C.

4. When applying the polarographic method, continuing reaction and hydrolysis effects in the polarographic solution must be considered (Figs. 4 and 5).

5. In an unbuffered solution, 4.0 M $CO(NH_2)_2$ + 4.0 M HCHO, initial pH 6.70 and at 20° C, no rapid initial reaction can be found. No theoretically significant reaction order is present; the order is approximately 1.6. (A number of repeated experiments of this kind, at different concentrations of the reactants and different initial pH, and also such carried out with the pH of the reaction solution kept constant, gave the same result.) (Fig. 8).

6. In buffered solutions and a urea-formaldehyde ratio of 1:1 at 20° C no theoretically significant reaction order is present and no initial reaction observed. The reaction order is approximately 1.6 (Fig. 8).

7. At the urea-formaldehyde ratio 1:1, in buffered solutions at 20° C, no obvious concentration dependence of the reaction rate can be seen (Fig. 9).

8. In buffered solutions containing 4.0 M $CO(NH_2)_2$ + 0.4 M HCHO a second order reaction occurs (Figs. 10—14).

9. The rate of the second order reaction in solutions containing an excess of urea is not obviously dependent on this excess or the concentration of the reactants. (At a low urea-formaldehyde ratio, 5:1, small deviations from linearity are present, showing a tendency to a non-linear relationship.) (Figs. 15 and 16).

10. The urea-formaldehyde reaction at 20° C in solutions containing an excess of urea is subject to an *acid-base catalysis*. Different acid and base ions show different catalysing power. An extremely small effect is present in the

case of borate ions (Fig. 17). The same effects were observed at a urea-formaldehyde ratio of 1:1.

11. The hydroxyl ion catalytic effect is not a simple function of the activity of this ion (Fig. 18).

The author wishes to thank the board of *Rydboholms A.B.* for permission to publish this paper, and is also indebted to Mr G. Johansson for his valuable assistance in the experimental work and to Professor N. Gralén and Dr. J. Lindberg for their kind interest.

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*iso*Thiocyanates XII. 3-Methylthiopropyl *iso*Thiocyanate (Ibervirin), a New Naturally Occurring Mustard Oil

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The previously unknown 3-methylthiopropyl *iso*thiocyanate (II) has been synthesized and its occurrence in glucosidic form in nature has been demonstrated. Paper chromatography has indicated the simultaneous presence in seeds of *Iberis sempervirens* L. of glucoiberin, glucoerucin and the new glucoside, for which the name *glucoibervirin* is suggested. From this seed material a mixture of 3-methylthiopropyl- and 4-methylthiobutyl-thiourea has been obtained after enzymatic hydrolysis, steam distillation and treatment with ammonia. The mixture has been quantitatively separated by a 100-transfer countercurrent distribution and the individual compounds identified on comparison with synthetic specimens.

In a previous paper of this series [1] 4-methylthiobutyl *iso*thiocyanate (I) was recognized as a constituent of glucoerucin, a new naturally occurring mustard oil glucoside. We now wish to adduce experimental evidence for the presence in nature of a structurally related glucoside, provisionally termed *glucoibervirin*, which gives rise to 3-methylthiopropyl *iso*thiocyanate (II) on enzymatic degradation.

$CH_3SCH_2CH_2CH_2CH_2NCS$ I

$CH_3SCH_2CH_2CH_2NCS$ II

$CH_3SCH_2CH_2CH_2NCS$ (O) III

$CH_3SCH_2CH_2CH_2NHCSNH_2$ IV

Seeds of *Iberis sempervirens* L. were previously investigated for mustard oil glucosides by paper chromatography [2]. Revised results [3] indicated the presence of three glucosides one of which possessed an R_F-value considerably lower than those of the other two. Upon repetition here, a similar paper-chromatographic pattern was observed. From its behaviour in various solvent

systems the spot of low R_F-value could safely be attributed to glucoiberin, a glucoside containing methylsulphinylpropyl *iso*thiocyanate (III), recently characterized by Schultz and Gmelin [3,4]. The glucoside chromatograms did not render possible, however, a correspondingly unambiguous allocation of structure to the two glucosides of higher R_F-values.

When seeds of *Iberis sempervirens* L. were crushed and treated with water and myrosinase a characteristic radish-like smell soon developed, ascribable to enzymatic liberation of volatile mustard oils which could be removed by steam distillation. Upon treatment of the distillate with ammonia a mixture of two thioureas was produced as inferred from paper chromatography according to our usual method [5]. In chloroform-water as solvent system the two compounds possessed R_{Ph}-values of 0.82 and 0.99, respectively. They appeared to be present in the ratio 3 : 2 as roughly judged from the chromatograms. The highest R_{Ph}-value coincided with the value of the thiourea derived from 4-methylthiobutyl *iso*thiocyanate (I), the radish-smelling oil which we recently showed to be present in seeds of various crucifers [1]. The other spot fell within the range of *sec*-butylthiourea which at first glance would seem to be an attractive possibility in view of the well-established occurrence in many plants of glucococh learin, a glucoside yielding *sec*-butyl *iso*thiocyanate on enzymatic cleavage. This possibility could be excluded, however, when it was found that *sec*-butylthiourea travelled on the paper at a much higher rate than the unknown thiourea in heptane:*n*-butanol:formic acid, a solvent system [6] which has often proved to be useful also for our purpose. A distillation experiment on seeds of *Iberis amara* L., the source of glucoiberin [3,4], demonstrated volatile *iso*thiocyanates to be absent. Hence, (III) can be excluded as responsible for thiourea spots originating from the volatile fraction.

In view of the occurrence in the seed sample of glucoiberin, producing (III) on enzymatic cleavage, the simultaneous existence of the reduced form (II) appeared to be a reasonable assumption. The hitherto unknown mustard oil was synthesized from 3-methylthiopropylamine and thiocarbonyl chloride and transformed into 3-methylthiopropylthiourea (IV). Upon paper chromatography in various solvent systems the latter was indistinguishable from the thiourea of the seed distillate possessing an R_{Ph}-value of 0.82.

On the assumption that (I) and (II) represented the volatile *iso*thiocyanates of *I. sempervirens* the partition ratios for their thioureas between water and chloroform were determined and found to be 0.47 and 1.35, respectively. With these values a calculation indicated that a 100-transfer counter-current distribution would render possible a practically quantitative separation of the two thioureas, permitting their individual isolation and identification (Fig. 1). This was born out experimentally when the thiourea-mixture from the distillation of 50 g of seeds was distributed in a Craig-apparatus. From plates Nos. 22—39 were isolated pure 4-methylthiobutylthiourea, identified on comparison with an authentic specimen. The contents of the vessels Nos. 50—60 afforded chemically homogenous 3-methylthiopropylthiourea (IV), identical with the synthetic sample. Paper chromatography disclosed the presence of small amounts of an additional thiourea in plates with the serial numbers 65—71. Due to its presence in extremely small amounts this third constituent escaped analytical detection before fractionation. Only after enrichment during the

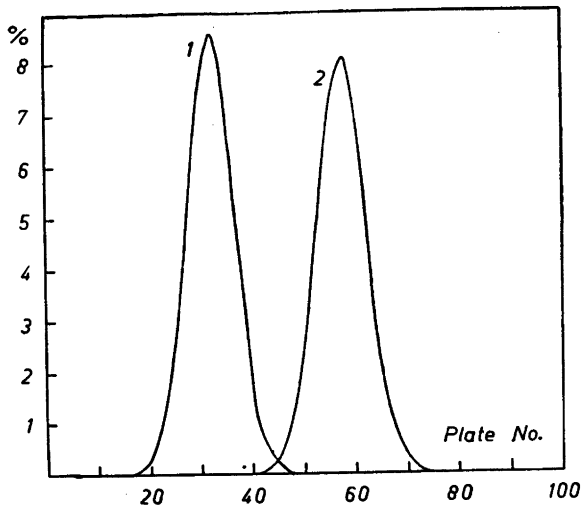

Fig. 1. Theoretical 100-plate distribution curves in water : chloroform of 1) 4-methylthiobutylthiourea with a partition coefficient of 0.47, and 2) 3-methylthiopropylthiourea (IV) with a coefficient of 1.35.

counter-current distribution could its identity as a thiourea-derivative of the elsewhere encountered 3-butenyl *iso*thiocyanate [7] be chromatographically verified.

The recognition of 3-methylthiopropyl *iso*thiocyanate as a natural constituent completes a series of mustard oils, differing only in the oxidation stage of the sulphur atom, *viz.*:

$CH_3SCH_2CH_2CH_2NCS$	Ibervirin
$CH_3SCH_2CH_2CH_2NCS$	Iberin [3,4]
$\downarrow$ O	
O $\uparrow$	
$CH_3SCH_2CH_2CH_2NCS$	Cheirolin [8]
$\downarrow$ O	

These types of *iso*thiocyanates have been encountered mainly in the genera *Iberis*, *Erysimum* and *Cheiranthus*. A survey of the contents of *iso*thiocyanate glucosides in various species of the two first genera has been made and will be published separately. A pronounced tendency to spontaneous oxidation of methylthio-derivatives to sulphoxides has been observed throughout the current studies. Consequently, the question arose to what extent the previously recognized sulphoxide and sulphone were artefacts, formed during isolation. Glucoiberin [3,4] was reported with a specific rotation of —55.3° whereas the corresponding value for sinigrin is —17.6°. From these data it can be inferred that

a considerable contribution to the total rotation resides in the sulphoxide-grouping in the side chain of glucoiberin. Here it should be remembered also that sulphoraphen (4-methylsulphinyl-3-butenyl *iso*thiocyanate), isolated by Schmid and Karrer [9] from seeds of *Raphanus sativus* L., possessed a considerable specific rotation. Hence, at least in some cases the sulphoxides and sulphones appear to be present as genuine plant constituents, very likely formed, however, by enzymatic oxidation of the corresponding sulphides during transportation and storage within the living cells. These problems are at present being further studied.

EXPERIMENTAL

All melting points are uncorrected and determined in capillary tubes in a water-bath. For chromatographic work redistilled solvents, boiling within 0.5°, have been employed.

Paper chromatography of glucosides. A methanolic extract of crushed seeds of *Iberis sempervirens* L. was applied to a strip of Schleicher and Schüll 2043 b paper and developed by the descending technique with *n*-butanol : acetic acid : water (4:1:3) for 4—6 hours. After spraying with silver nitrate and heating [2,3], three distinct spots appeared one of which was of low R_F-value and indistinguishable from glucoiberin, simultaneously run as a control substance. The two other spots appeared within the region of glucocochlearin and glucotropaeolin, respectively. A parallel run in pyridine : amyl alcohol : water (30:35:30) served to confirm the identity of the glucoiberin-spot.

Another seed sample was treated with myrosinase, steam-distilled and the volatile *iso*thiocyanates of radish-like smell transformed into thioureas according to our usual method [10]. Descending paper chromatography in chloroform : water [5] on Whatman paper No. 1, followed by spraying with Grote's reagent revealed two spots with R_{Ph}-values of 0.82 and 0.99. The latter coincided with the spot of 4-methylthiobutylthiourea [1] as well in this solvent system as in heptane : *n*-butanol : formic acid (20:15:20) [6]. A provisional interpretation of the 0.82-spot as due to *sec*-butylthiourea required revision when it was found that the butyl-derivative possessed an R_{Ph}-value of 1.15, compared to the value 0.51 for the *Iberis*-spot, when both were run in the formic acid system.

Synthesis of 3-methylthiopropyl isothiocyanate *(II) and -thiourea (IV).* To a cooled solution of thiocarbonyl chloride (8.2 g) in chloroform (60 ml) was added a solution of 3-methylthiopropylamine [11] (7.2 g) in water (45 ml). 100 ml of 1.4 N NaOH were slowly added with vigorous shaking and after half an hour the chloroform layer was separated, dried and the solvent removed *in vacuo*. Distillation under diminished pressure afforded the *iso*thiocyanate, b. p. 120.5—122°/12 mm. Redistillation gave an analytically pure product as a colourless liquid with a pronounced smell of radish (5.5 g, 54 %); b. p. 116°/9 mm. (Found: C 40.35; H 5.98; N 9.64. Calc. for $C_5H_9NS_2$: C 40.77; H 6.16; N 9.51).

The *iso*thiocyanate was dissolved in ethanol and excess of conc. aqueous ammonia was added. After 4 days at room temperature the reaction mixture was concentrated *in vacuo*, leaving an oil which rapidly crystallized. The thiourea (IV) was recrystallized twice from water containing a little ethanol, and finally from ethyl acetate and pentane as thin, hexagonal plates; m. p. 66—67°. (Found: N 16.98; S 39.08. Calc. for $C_5H_{12}N_2S_2$: N 17.06; S 39.03).

Counter-current distribution. A portion (50.2 g) of finely ground seeds of *Iberis sempervirens* L. was refluxed with a mixture of ethanol (100 ml) and ligroin (200 ml, b. p. 60—100°) for 20 minutes and the extraction repeated with petroleum ether (250 ml) and ethanol (50 ml) for 15 minutes. The dried powder was then treated with water (200 ml) and a cell-free myrosinase-preparation (2.0 ml) and left at room temperature overnight. The volatile *iso*thiocyanates were removed in a stream of steam and collected in a receiver containing 25 % ammonia (125 ml). After standing at room temperature for 24 hours the solution was taken to dryness *in vacuo* (bath temperature not exceeding 50°). The yellow, viscous residue (909 mg) was brought into solution in chloroform by the addition of some ethanol. The solution was transformed to plates Nos. 0—2 of an all-glass Craig counter-current apparatus, preloaded with water-saturated chloroform in the 100 plates, each holding 40 ml of both the lower and upper phase. 100 transfers were made; at the end of the

distribution the fractions were collected individually and each five concentrated and investigated paperchromatographically. Aside from a very small overlapping in plates Nos. 40—45 a quantitative separation was achieved in agreement with the calculated curves of Fig. 1. The contents of plates Nos. 22—39 were pooled and concentrated to dryness *in vacuo*, giving a semi-crystalline mass (275 mg). Two recrystallizations from ethyl acetate and petroleum ether afforded thin, colourless plates (116 mg); m. p. 51°, alone or in admixture with authentic 4-methylthiobutylthiourea. (Found: C 40.34; H 7.80; N 15.85. Calc. for $C_6H_{14}N_2S_2$: C 40.42; H 7.91; N 15.72). Plates Nos. 50—60 were pooled and worked up in a similar way. The crude product (229 mg) was recrystallized twice from ethyl acetate and petroleum ether and gave shiny, rhombic plates (150 mg); m. p. 67° alone or when mixed with the synthetic 3-methylthiopropylthiourea (IV) above. (Found: C 36.20; H 7.17; N 17.22. Calc. for $C_5H_{12}N_2S_2$: C 36.55; H 7.37; N 17.06).

Paper chromatography in chloroform of the residues from plates Nos. 65—71 disclosed the presence of very small amounts of an additional thiourea with an R_{Ph}-value of 0.62. Its identity as 3-butenylthiourea is regarded as established by this value.

We are very thankful to *Dansk Sojakagefabrik* Inc. for the loan of a Craig-apparatus. Thanks are due also to the Botanical Gardens of Berlin-Dahlem, Frankfurt a. M. and Copenhagen for furnishing us with seed samples. Valuable assistance of Miss E. Borch in the syntheses is acknowledged. Microanalyses by Mr. P. Hansen.

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Studies on Liver Alcohol Dehydrogenase

III. The Influence of pH and Some Anions on the Reaction Velocity Constants

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By the aid of fluorescence measurements the initial reaction velocity constants in the liver ADH ** system have been determined in the pH range 5.3 to 10. Our previously postulated reaction mechanism appears to be followed as indicated by three independent lines of evidence:

1. The kinetical data when applied for calculating the equilibrium constant K of the whole system using the equations derived from our postulate gave values fairly close to the true dissociation constant, 10^{-11}, in the whole pH range.
2. The relationship between the dissociation constants of the oxidized and the reduced enzyme · coenzyme complex agreed with previous equilibrium data with excess of enzyme.
3. Two of the velocity constants had previously been determined spectrophotometrically in single reaction steps and agreed with our present data from the overall reaction velocities.

On the basis of this reaction mechanism, the significance of the different velocity constants and their variations with pH has been discussed.

Different anions were found to have remarkable effects on some of the velocity constants. In some cases, these effects were of opposite sign for the oxidized and reduced enzyme complexes and the oxidation-reduction potential could be calculated to be strongly dependent on the anion concentration. The significance of these findings is discussed. Liver ADH has been found to contain two atoms of zinc per molecule.

In the previous papers of this series [1,2] some data were given for the velocity constants in the reversible system

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** *Abbreviations.* DPNH: reduced, and DPN: oxidized diphosphopyridine nucleotide; ADH: ½ molecule of alcohol dehydrogenase; ald: acetaldehyde; alc: ethanol; x, x^1, e, a and a^1: concentrations of corresponding reactants.

$$\overset{x}{\text{DPNH}} + \overset{e}{\text{ADH}} \underset{k_2\ (\text{sec}^{-1})}{\overset{k_1\ (\text{M}^{-1} \times \text{sec}^{-1})}{\rightleftharpoons}} \text{DPNH} \cdot \text{ADH} \qquad (1)$$

$$\text{DPNH} \cdot \text{ADH} + \overset{a}{\text{ald}} + \text{H}^+ \underset{k_6\ (\text{M}^{-1} \times \text{sec}^{-1})}{\overset{k_4\ (\text{M}^{-2} \times \text{sec}^{-1})}{\rightleftharpoons}} \text{DPN} \cdot \text{ADH} + \overset{a^1}{\text{alc}} \qquad (2)$$

$$\text{DPN} \cdot \text{ADH} \underset{k_5\ (\text{M}^{-1} \times \text{sec}^{-1})}{\overset{k_3\ (\text{sec}^{-1})}{\rightleftharpoons}} \overset{x^1}{\text{DPN}} + \text{ADH} \qquad (3)$$

It was shown that the experimental data were reasonably compatible with the reaction scheme 1—3. This scheme requires a compulsory order of reactions: the coenzyme first combining with the enzyme, and the complex then reacting with the substrate. Furthermore, if a ternary complex is formed, the dissociation velocity of the substrate product must be high compared with the dissociation velocity of the coenzyme-enzyme. The older data were neither accurate, nor extensive enough to warrant definite conclusions as to the validity of this reaction scheme. The problem could be settled only by more accurate determination of the six velocity constants, and the relationship between these and the equilibrium constant.

Alberty [3] has derived formulas for different cases of reaction sequences for systems consisting of enzyme, coenzyme and substrate. When inserting our values into his formulae he found, that some of the data fitted with the reaction scheme 1—3, but others did not. We have now shown that this discrepancy was mainly due to the inaccuracy of some older data and the uncertainties in the determinations of some Michaelis constants.

The spectrophotometric methods used in 1951 did not permit us to work at sufficiently low concentrations of the reactants or at high enough acidities to get a sufficiently complete survey of the influence of pH on the magnitude of the reaction velocity constants. This is now possible by using fluorimetry instead of spectrophotometry [4]. The strong anion effects on the association and dissociation reactions of coenzyme and protein in the old yellow enzyme (O. Y. E.) [5] prompted us to make a corresponding investigation of the ADH system.

MATERIALS AND METHODS

DPNH gives a bluish-white fluorescence [6] that is not quenched by coupling to ADH, whereas DPN does not fluoresce. It is thus possible to follow the oxidation of DPNH or reduction of DPN by fluorescence measurements.

The experiments were carried out in 1 cm cuvettes, in a total volume of 3 ml. The reactions were always started by adding a suitable amount of ADH from a stirring rod at $t = 0$. In our apparatus, the intensity of the fluorescence of DPNH was about 4 % of the fluorescence of flavin mononucleotide (FMN) on an equimolar basis. Still the sensitivity is very high compared to spectrophotometry. A micromolar solution of DPNH in 1 cm cuvettes, when using sensitivity 10 inches per 0.1 volts, entrance slit 1 mm, and exit slit 6 mm, gave deflections of around 5 inches on the recorder. With larger entrance slits the inflections increase proportionally. For a comparison with spectrophotometry it should be remembered that the light absorption of the same solution at 340 mμ would be as low as $\log I_0/I = 0.006$. The practical limit of the sensitivity of the apparatus is set by the

fluorescence of the buffer solutions. Because of the high sensitivity of the apparatus, it was feasible to determine much smaller amounts of ethyl alcohol or acetaldehyde than with spectrophotometry. From the rate of the oxido/reduction of DPN(H) in the ADH system we have been able to determine quantitatively concentrations of alcohol and acetaldehyde down to 0.5×10^{-6} M.

The fluorescence of DPNH is proportional to the concentration up to about 20×10^{-6} M. Above this concentration, the 340 mμ band absorbs the incident light appreciably, so that corrections must be made.

Crystalline ADH was prepared according to the method of Bonnichsen [7]. The concentration of ADH was determined both from optical density at 280 mμ, and from kinetic measurements. Thus, k_2 at pH 7.15, 23.5°, was found to be 1.6 sec^{-1} for a homogeneous preparation of ADH. The maximum rate for the oxidation of alcohol was consequently determined for all preparations under these conditions, and the concentration of ADH determined on the basis of $k_2 = 1.6$ sec^{-1}. There was always agreement between the kinetic and spectrophotometric measurements.

DPN: A preparation which was 75 % pure, was made according to Neilands and Åkeson [8] in this laboratory.

DPNH was produced enzymatically, as described by Bonnichsen [9]. The preparation was lyophilized.

Buffers. The remarkable effects of anions observed on the O.Y.E. system [5], prompted us to make a careful selection of buffers for studying the pH effects independent of anion effects. The buffers were tested at different concentrations to make sure that they did not interfere appreciably with the reaction velocity. 0.05 M citrate was chosen at pH 5.3, phosphate, ionic strength 0.1, at pH 6 and 7, 0.1 M glycyl-glycine at pH 8, and 0.1 M glycine at pH 9 and 10. It is to be noticed that 0.05 M pyrophosphate, which has been used in previous experiments [1,2], inhibits the reduction of acetaldehyde appreciably at pH 9, so that at this pH the older values are not strictly comparable to the new ones.

CALCULATIONS

We have in this investigation exclusively determined the initial reaction velocities at $t \sim 0$. The reaction scheme 1—3 was found [2] to lead to the following equations:

Reduction of aldehyde by DPNH:

$$\frac{1}{e} \times \frac{dc}{dt} = \frac{1}{\frac{1}{k_1 x} + \frac{1}{k_4 [H^+] a}\left((1 + \frac{k_2}{k_1 x}\right) + \frac{1}{k_3}} \qquad (4)$$

Oxidation of ethanol by DPN:

$$\frac{1}{e} \times \frac{dc}{dt} = \frac{1}{\frac{1}{k_5 x^1} + \frac{1}{k_6 a^1}\left(1 + \frac{k_3}{k_5 x^1}\right) + \frac{1}{k_2}} \qquad (5)$$

In order to determine all six velocity constants at a given pH, ion concentration and temperature it is necessary to make four sets of experiments varying the reactants according to Table 1.

In most circumstances it is possible to obtain a reaction velocity low enough to be practically constant during the first 30—60 seconds by making the ADH concentration suitably low (see Fig. 1). Fig. 2 shows a reaction cycle with high [ald] and low [DPNH] (= 1.0 μM). Here the enzyme concentration was unsuitably large for obtaining a reliable tangent to the curve at $t = 0$; but,

Table 1. Variation in the concentrations of the reactants.

Expt. No.	[DPNH]	[ald]	[DPN]	[alc]
1	Varied	High	—	—
2	High	Varied	—	—
3	—	—	Varied	High
4	—	—	High	Varied

as would be expected, the reaction under the conditions used was of first order, so that confirmation of the slope at $t = 0$ could be obtained from other points on the curve.

The results from the four series of experiments when plotted according to Lineweaver and Burk [10] gave straight lines within the limits of error. However, alcohol concentrations >10 mM caused inhibition and were avoided.

We used the following procedure for calculating the velocity constants.

Two independent values for k_3 were obtained from the experiments Nos. 1 and 2, Table 1. e/V'_{max} is the intercept of the Lineweaver-Burk plot with the ordinate. In order to obtain k_3, which is equal to V_{max}/e at [DPNH] $= \infty$ and [ald] $= \infty$ [2] the value for e/V'_{max} from expt. No. 1 was corrected to infinite [ald]. Thus

$$k_3 = \frac{e}{V'_{max}}\left(1 + \frac{K_{m,ald}}{[ald]}\right) = \frac{V_{max}}{e} \qquad (6a)$$

and correspondingly from expt. 2

$$k_3 = \frac{V''_{max}}{e}\left(1 + \frac{K_{m,DPNH}}{[DPNH]}\right) = \frac{V_{max}}{e} \qquad (6b)$$

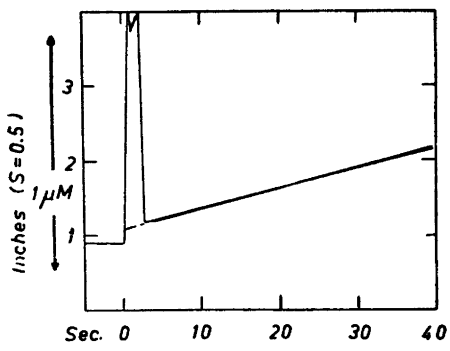


Fig. 1. Record of the increase in fluorescence after adding ADH at $t = 0$ to DPN and alcohol. pH 7.15. [ADH] $= 1.9 \times 10^{-8}$ M; [DPN] $= 4.8 \times 10^{-6}$; [alc] $= 7.7 \times 10^{-3}$. $S = 0.5$, slits 3 and 6 mm.

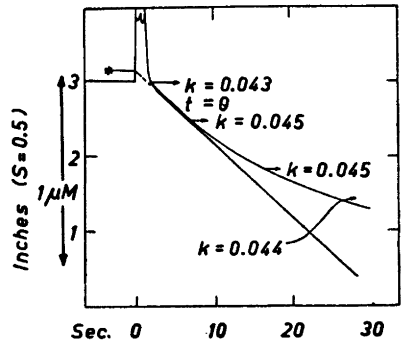


Fig. 2. Record of the decrease in fluorescence after adding ADH to DPNH and acetaldehyde. pH 7.15. ADH $= 1.65 \times 10^{-8}$ M; [DPNH] $= 1.0 \times 10^{-6}$, [ald] $= 3.8 \times 10^{-3}$.

k_2 is obtained from expts. 3 and 4 in an analogous manner. It should be noticed that this extrapolation to V_{max}/e is permissible only if the concentration of the reactant that is kept "high" and constant is considerably higher than K_m. This is also a prerequisite for obtaining approximately true Michaelis constants for the varied reactant.

We then chose a pair of points, one from each of the experiments 1 and 2, which gave the same e/V. The concentration of the aldehyde was then a_1 and a_2, the concentration of DPNH $= x_1$ and x_2. Under these conditions we found

$$k_4[H^+] = \frac{k_1 x_1 x_2 \ (a_1 - a_2) + k_2(a_1 x_1 - a_2 x_2)}{a_1 a_2 \ (x_1 - x_2)} \qquad (7)$$

Since k_2 is known the value for k_1 can thus be found from eq. 4, and k_4 $[H^+]$ from eq. 7.

k_5 and k_6 were obtained from experiments Nos. 3 and 4 (Table 1) in an analogous way. Many different constants can be derived from the velocity constants, as indicated by Table 2.

Table 2. Dependance of Michaelis and equilibrium constants on the kinetic constants, assuming the reaction sequence 1—3 to be valid.

Michaelis constants, K_m				Dissociation constants for holoenzyme			Equilibrium constant
							K
DPNH	ald	DPN	alc	Reduced D_{red}	Oxidized D_{ox}	D_{ox}/D_{red}	
$\frac{k_3}{k_1}$	$\frac{k_3}{k_4[H^+]}$	$\frac{k_2}{k_5}$	$\frac{k_2}{k_6}$	$\frac{k_3}{k_1}$	$\frac{k_3}{k_5}$	$\frac{k_3 k_1}{k_5 k_2}$	$\frac{k_2 k_5 k_6}{k_1 k_3 k_4}$

RESULTS

The new data are summarized in the Tables 3 and 4, and Fig. 3 shows the Lineweaver-Burk plots of some experiments in phosphate, phosphate + chloride and phosphate + formate at pH 7.15. We had now the possibility to check the validity of the proposed reaction scheme in the whole pH range 5.3 to 10.

Michaelis constants

It is seen from Tables 3—4 that the agreement between the experimentally observed values for K_m and the values calculated from the velocity constants is good in the experiments with DPNH and aldehyde. Greater deviations are found in the experiments with DPN and alcohol at pH 5.3 to 7.15. In some cases this is probably because the Michaelis constants are so high that the reactant which was held constant could not be used in great enough excess.

Table 3. Reaction velocity constants at 23.5° Ionic Strength of all buffers (Na-Salts) = 0.1

Velocity constants	pH 5.3 Citrate	pH 6.05 Phos-phate	pH 7.15 Phos-phate	pH 7.15 Phos-phate +0.15 M NaCl	pH 7.15 Phos-phate +0.015 M formate	pH 8.0 Glycyl-glycine	pH 9.0 Glycine	pH 10 Glycine
$10^{-6} \times k_1$	1.5	3.3	3.7	1.8	3.7	3.7	2	0.5
k_2	0.28	0.65	1.6	2.8	1.6	1.6	1.9	2
$10^{-6} \times k_4$ $[H^+]$	0.036	0.036	0.24	0.21	0.24	0.069	0.012	0.0008
$10^{-13} \times k_4$	0.00073	0.004	0.34	0.30	0.34	0.69	1.2	0.8
$10^{-4} \times k_6$	0.011	0.039	0.35	0.19	0.12	0.85	0.35	0.09
k_3	6	17	37	18	9.5	20	5	0.7
$10^{-6} \times k_5$	0.011	0.075	0.30	0.14	0.30	0.28	0.18	0.04

However, this is not likely to be the whole explanation of the discrepancies. Some deviation from the postulated mechanism appears to occur within this region. This deviation could be connected with the fact that alcohol in concentrations as low as 10 mM already has an inhibitory effect.

The new data for the K_m are in fair agreement with the older data with two exceptions; K_m for aldehyde has now been found to be 270 μM at pH 8, 400 μM at pH 9, whereas in the previous work we obtained 52 and 2 300, respectively. The difference may be due to the use of non-inhibiting buffers in the present experiments. In this connection it should be mentioned that for some unknown reason we had to repeat the experiments with aldehyde at pH 9 several times to get reliable values. The experimental data at pH 10 are definitely less certain than the others.

Table 4. Michaelis constants, equilibrium constants, and oxidation-reduction potentials calculated from kinetic constants.

Michaelis and equilibrium constants	pH 5.3	pH 6.05	pH 7.15	pH 7.15 NaCl	pH 7.15 NaCOONa	pH 8	pH 9	pH 10
k_3/k_1 μM	4.0	5.1	10	10	2.6	5.4	2.5	1.4
K_m, DPNH exptl. »	3.5	4.7	10	10	2.5	5.4	3	2.1
k_3/k_4 H^+ »	170	470	150	86	40	290	400	880
K_m, ald., exptl. »	270	410	110	50	41	270	400	800
k_2/k_5 »	25	9	5	20	5	5.7	10.5	50
K_m, DPN, exptl. »	77	25	10	33	10	5.0	11	40
k_2/k_6 »	2 700	1 700	460	1 500	1 330	190	550	2 200
k_m, alc., exptl. »	4 600	2 500	590	2 100	2 900	250	600	2 000
$K = \frac{k_2 k_5 k_6}{k_1 k_3 k_4} \times 10^{11}$	0.50	0.93	0.37	0.77	0.48	0.73	0.95	2.6
$D_{ox}(= k_3/k_5)$ μM	550	226	123	129	32	71	28	18
$D_{red}(= k_2/k_1)$ »	0.19	0.20	0.43	1.6	0.43	0.43	0.95	4
D_{ox}/D_{red} —	2 900	1 150	285	83	74	165	29	4.5
E_0 (holoenzyme), V	—0.126	—0.156	—0.204	—0.219	—0.221	—0.234	—0.290	—0.342

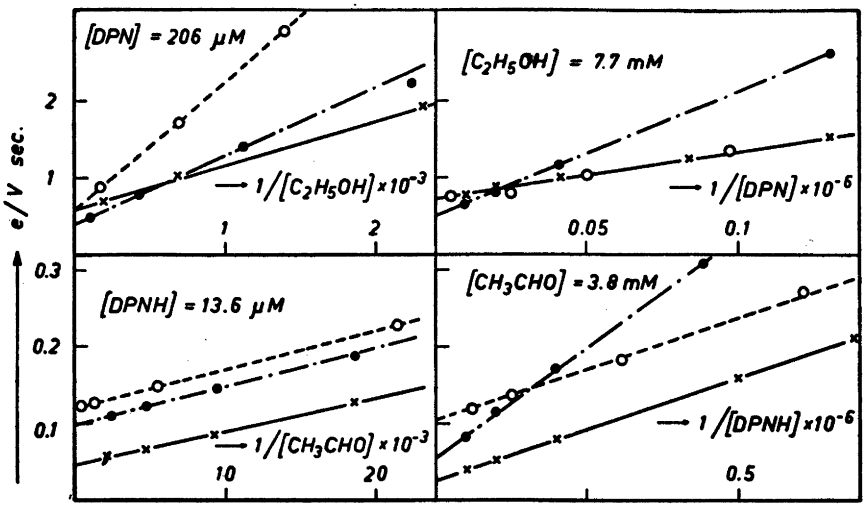


Fig. 3. Lineweaver-Burk plots of three sets of experiments according to Table 1. pH = 7.15, 23° C.

× *Phosphate buffer ionic strength = 0.1*
● » » » » = » + *0.15 M NaCl*
○ » » » » = » + *0.015 M Na formate*

Dissociation velocity constants

A comparison of the new and the old data for k_1 and k_2 reveals that the values between pH 6.8 and 9 agree fairly well, but at pH 10 we now found $k_2 = 2$ and $k_3 = 0.7$, whereas 5 and 4.5 was obtained in the previous work.

Association velocity constants

The present value for k_1 at pH 7.15, 3.7×10^6 $M^{-1} \times sec^{-1}$, agrees remarkably well with the older value 4×10^6 obtained by the independent method of rapid spectrophotometry [2] of the band shift 340—325 mμ in the direct reaction DPNH + ADH → DPNH · ADH.

In the previous work k_5 was computed in an indirect way to be 2×10^6 $M^{-1} \times sec^{-1}$. This value is far too high as compared with the present value at pH 7, 0.3×10^6. The discrepancy is explained by the fact that the old value was based upon an uncertain value for D_{red}, obtained by spectrophotometry of very dilute (~1 μM) solutions of DPNH and ADH (for definition of D_{red}, *cf.* Table 2 and Ref.[1]). Thus contrary to our previous conclusion, k_5 is appreciably lower than k_1, around 12 times at neutral or alkaline reaction, still more in acidic medium.

The new value for k_4 [H+] at pH 7.15, 0.24×10^6 $M^{-1} \times sec^{-1}$, agrees with the old value for k_4 *, 0.22×10^6. This is not surprising, since both values

* In the previous work [2] k_4 was taken as $k_4 = \frac{dc}{dt} \times \frac{1}{[DPNH \cdot ADH][ald]}$; in the present we have preferred to design $k_4 = \frac{dc}{dt} \times \frac{1}{[H^+][DPNH \cdot ADH][ald]}$. The previous k_4 is thus = k_4 [H+] in the present paper.

Table 5. Our kinetical data inserted into three formulas representing reaction sequences.

pH	K_{exp} $\times 10^{-11}$	Two complexes formed. Mech. proposed by Theorell &Chance[2] = Mech. 9 of Alberty [3] $K^* = \frac{k_2 \cdot k_5 \cdot k_6}{k_1 \cdot k_3 \cdot k_4} \times 10^{-11}$	Three or four complexes formed Mech. 7 of Alberty [3] $K = \frac{k_3 \cdot k_5 \cdot k_6}{k_1 \cdot k_2 \cdot k_4} \times 10^{-11}$	Substrate or co-enzyme is oxidized/ reduced by enzyme Mech. 14 of Alberty [3] $K = \frac{k_5 \cdot k_6}{k_1 \cdot k_4} \times 10^{-11}$
5.3	0.86	0.5	230	10.7
6.0	0.86	0.95	640	24.4
7.1	0.86	0.37	197	8.6
8.0	0.86	0.73	114	9.1
9.0	0.86	0.95	6.6	2.5
10.0	0.86	2.6	0.32	0.91

* Alberty's expression is $K = \left(\frac{V_f}{V_r}\right)^2 \frac{K_{Ald} \cdot K_{DPNH}}{K_{Alc} \cdot K_{DPNH}}$, where V_f = max. velocity for the oxidation of alcohol (= k_3) and V_r = max.velocity for reduction of aldehyde (= k_5). When our expressions for Michaelis constants are inserted into the above equation $\left(\frac{V_f}{V_r}\right)^2 \frac{K_{Ald} \cdot K_{DPNH}}{K_{Alc} \cdot K_{DPN}}$ becomes equal to $\frac{k_2 \cdot k_5 \cdot k_6}{k_1 \cdot k_3 \cdot k_4}$. The expressions for Mech. 7 and 14 of Alberty [7] are formed in analogous ways.

were determined from initial velocities with low concentrations of ADH. But it should be pointed out that these values are of the same order of magnitude as the value determined by rapid spectrophotometry of the complex, DPNH · ADH + acetaldehyde (k_4 [H$^+$] $\sim 0.1 \times 10^6$ M$^{-1} \times$ sec^{-1}). Moreover, the last value was certainly too low, since we know from the present work that D_{red} is considerably higher, and [DPNH · ADH] must thus have been lower than calculated in the previous work. The rather satisfactory agreement between the values for k_4 [H$^+$] with acetaldehyde, obtained by two independent methods must be regarded as a strong support of the postulated reaction mechanism.

k_6 was not calculated from the old data [1,2].

The equilibrium constant

K, as calculated from the six initial velocity constants (see Table 4) is sometimes higher, sometimes lower than our previous mean value from equilibrium determinations, 0.86×10^{-11}. It should be pointed out that considerable sources of error exist. For example, it was not possible to make all the four sets of experiments indicated in Table 1 on the same day. For technical reasons the experiments with DPNH and aldehyde had to be made on one day, the experiments with DPN and alcohol on another. As seen from the formula $K = \frac{k_2 k_5 k_6}{k_1 k_3 k_4}$ all the constants in the numerator derive

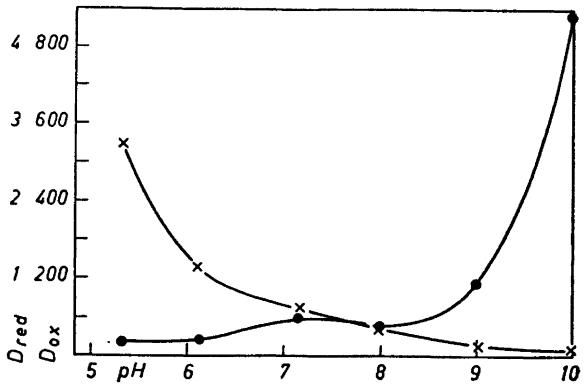


Fig. 4. Variation of D_{ox} and D_{red} with pH.
× D_{ox} μM
● D_{red} »

from the latter sets of experiments, all the constants in the denominator from the former. Errors in the determination of the enzyme concentration, or the calibration of the deflections of the recorder will therefore appear elevated to a potency of three in K (and two in D_{ox}/D_{red}). Therefore we venture that the agreement is as good as could be expected, and adds evidence to our postulated reaction sequence. This is clearly evident when we compare our new values with those calculated from Alberty's [3] main alternative mechanisms, see Table 5.

Alberty [3] found our old data at pH 7 and 8 to agree best with his mechanism 14, at pH 9 and 10 with mechanism 9 (the same mechanism as postulated in this paper). Our present data, on the basis of the accurately determined kinetical constants agree from pH 5.3 to 10 by far best with mechanism 9. It should be noticed how very sensitive the value of K becomes to errors in k_2 and k_3 (= V_f and V_r, respectively) when Alberty's formula for mechanism 9 is used, because it contains (V_f/V_r) [3]. Alberty used our old value $k_2 = 1.1$ at pH 7, whereas our present value at pH 7.15 is $k_2 = 1.6$. Already this discrepancy accounts for threefold error in K.

The dissociation constants of the enzyme-coenzyme compounds

D_{ox} $(= \frac{k_3}{k_5})$ decreases rapidly and continuously with increasing pH, whereas D_{red}, though less markedly, moves the opposite way (see Fig. 4). Possible reasons for this peculiar fact will be discussed later.

As a consequence of this opposite pH-dependence of the dissociation constants, the ratio D_{ox}/D_{red} is very strongly influenced by pH. This was already shown in the equilibrium data obtained in the previous work, from which this ratio was found to be approximately 200, 100, 10 and 3 at pH 7, 8 and 10 respectively. The present data, based upon initial velocity measurements and therefore independent of the equilibrium measurements, confirm the older

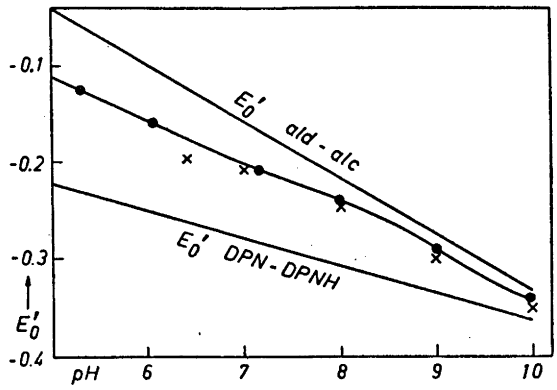


Fig. 5. Oxidation reduction potential of coenzyme-enzyme complex at different pH-values, calculated from D_{ox}/D_{red}.

● *new data*
× *equilibrium data from 1951* [1]

data (from pH 7 to 10). The new values are a little higher (see Table 4), but this is to be expected, because we can now see from our new data for D_{ox} and D_{red} that the excess of ADH was not quite sufficient in the equilibrium experiments of 1951 to make the concentrations of free DPN and free DPNH negligible.

As pointed out before [1] the oxidation-reduction potential of the enzyme-coenzyme complex can be calculated from the formula E_0' (enzyme-coenzyme) $= E_0''$ (coenzyme) $+ \frac{RT}{nF} \ln \frac{D_{ox}}{D_{red}}$. The oxidation-reduction potential of the enzyme-coenzyme compound at different pH-values, using the new values for D_{ox}/D_{red} are plotted in Fig. 5, where the older values are also included for comparison. The new values do not differ very much from the older; and a break in the curve from the slope 0.030 V/pH to 0.060 V/pH occurs, as before, near pH 7.8, indicating a titrable group in DPN · ADH that has not the same pK' in DPNH · ADH.

pH-dependence of the velocity constants

Fig. 6 shows the values for k_2 as a function of the pH. They coincide rather well with a monovalent dissociation curve with p$K' = 6.3$ and varying in height from the hypothetical value 0, at strongly acidic reaction to ~ 1.8 at alkaline reaction. In the presence of excess of both DPN and alcohol, k_2 determines the overall activity. The enzyme system does not show any "pH-optimum" under these experimental conditions but a fairly constant reaction rate in the whole pH-range 7 to 10.

k_3 can be interpreted in terms of two monovalent dissociation curves with pK':s at 6.4 and 7.8 (see Fig. 7). The hypothetical maximum of $k_3 = 60$ is never reached because of the interaction between the two groups. The plot of k_3 *versus* pH has the bell-shape which is so often observed when enzyme

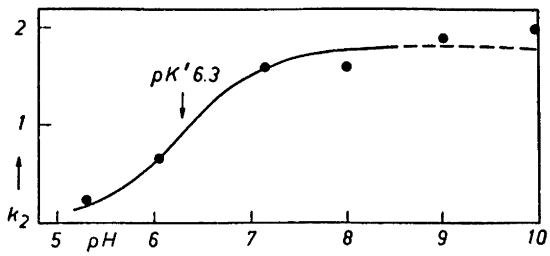


Fig. 6. Variation of k_2 *with pH.*

activity is measured as a function of pH, and which Michaelis attributed to titrable groups of importance for the activity. In our system k_3 is decisive for the overall activity of the enzyme under certain conditions, that is, when both DPNH and aldehyde are in excess. The ADH system under these conditions has its "pH-optimum" at pH 7.1.

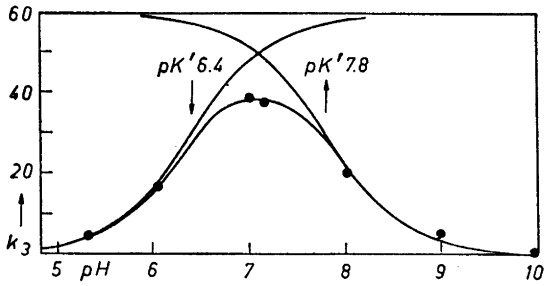


Fig. 7. Variation of k_3 *with pH. The values are close to a difference curve between two monovalent dissociation curves with* pK:s *6.4 and 7.8.*

k_1 and k_5 are both maximal between pH 7 and 8 (see Fig. 8) and both decrease proportionally towards the alkaline side, the pK' being approximately 9 in both cases. It should be noticed, however, that k_1 is here more than 10 times as great as k_5. In the acidic range k_5 begins to decrease at a higher pH (pK' = 6.5) than k_1 (pK' = 5.5), so that at pH 5.3 k_1 is roughly 100 times as

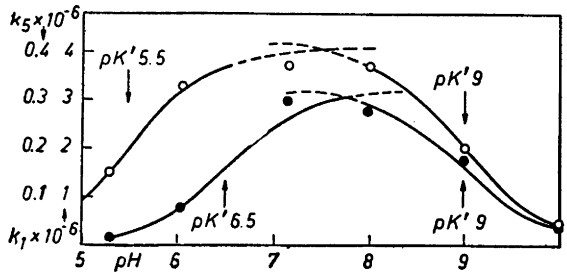


Fig. 8. Variation of k_1 (○) *and* k_5 (●) *with pH.*

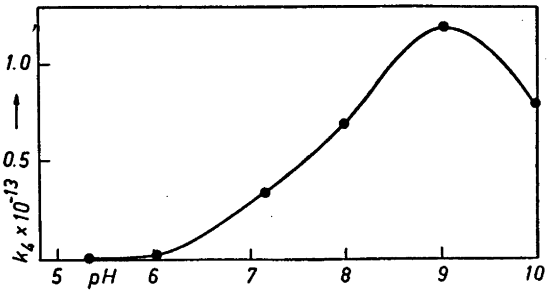


Fig. 9. Variation of k_4 *with pH.*

great as k_5. The "pH optimum" of ADH with excess of acetaldehyde and small amounts of DPNH occurs slightly above pH 7. The same should be the case for the reverse reaction: excess of alcohol, small amounts of DPN.

k_4 versus pH has a more complicated shape (see Fig. 9). It increases from very low values at pH 5.3 and 6 towards the alkaline side, where a flat maximum is found near pH 9. The plot cannot be interpreted in terms of a monovalent dissociation curve.

The pH-optimum for ADH with excess of DPNH and small amounts of acetaldehyde is again found at pH 7, because k_4 $[H^+]$ is then rate-limiting. The maximal reaction velocity in the system ADH + DPNH + acetaldehyde is thus always found near pH 7, though it should be noticed that the maximum is *narrow* with [DPNH] = ∞, [ald] small; *broad* with [DPNH] small, [ald] =∞; and intermediate with both [DPNH] and [ald] = ∞.

k_6 varies along a bell-shaped curve with a maximum near pH 8 (this was already evident in our previous paper (Fig. 8, curve III) and the branches fitting fairly well with monovalent dissociation curves of pK 7.5 and 8.8; see Fig. 10.

The effect of anions

A survey of the effect of different anions on the liver-ADH system revealed strong effects of such anions as chloride, bromide, nitrate and sulfate. This

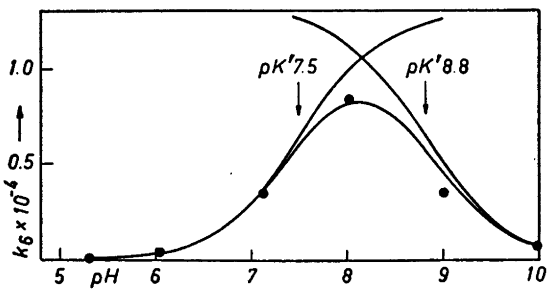


Fig. 10. Variation of k_6 *with pH. The experimental values are close to a difference curve between two monovalent dissociation curves with* pK:s *7.5 and 8.8.*

was expected from the work on the old yellow enzyme. But it was surprising to find that both acetate and formate, which were inactive in the O.Y.E. system, had strong effects on the liver-ADH system. In contrast, versene, which strongly inhibits the reassociation of the old yellow enzyme, did not affect the ADH system. This is shown in Table 6.

Table 6. Influence of some sodium salts, 0.15 M, on the reaction velocity, V/e sec^{-1}, in the ADH—DPN—DPNH system. pH = 7.1, phosphate buffer, ionic strength 0.1, 23.5°.

	ADH [1] = 0.13 μM CH_3CHO = 2 100 » DPNH = 9.6 »	ADH [1] = 0.13 μM C_2H_5OH = 2 300 » DPN = 8 »
Salt	*V/e* sec^{-1}	*V/e* sec^{-1}
Glycine	15	0.32
Phosphate	8.7	0.26
» + versene	8.9	—
Chloride	3.5	0.097
Sulphate	2.3	0.059
Nitrate	1.6	0.013
Bromide	0.13	0.055
Formate	0.17	0
Acetate	—	0.059

In the present investigation we determined the six velocity constants in the presence of 0.15 M sodium chloride and 0.015 M sodium formate at pH 7.15. The results are shown in Fig. 3 and Tables 3 and 4. 0.15 M chloride decreased the rate of association of DPN and DPNH with ADH (k_1 and k_5) to about half, and increased k_2, the dissociation velocity constant of the reduced complex. A similar increase in dissociation velocity by chloride ions was observed in O.Y.E. However, chloride decreased the rate of dissociation of the DPN·ADH complex (strong decrease in k_3) and such an effect was not observed in the O. Y. E. system by any anions. This suggests that other types of linkages may exist between DPN(H) and ADH than between FMN and the apoenzyme of O.Y.E. 0.015 M formate was found to affect only two of the six constants, k_3 and k_6. This means that formate reacts with the DPN·ADH complex, but neither with DPN or ADH before the complex is formed nor with the reduced complex. Formate may enter into the binding site for ethanol in DPN·ADH, since it decreased k_6 competitively. Formate had a similar effect on the yeast alcohol dehydrogenase system.

DISCUSSION

Our postulated reaction mechanism according to eqs. 1—3 has now been found to fit rather well with new experimental data over the whole range of pH from 5.3 to 10. The mechanism apparently does not take into consideration the formation of enzyme-substrate compounds. This may seem surprising

especially in view of the works of Kaplan *et al.*[11] and Vennesland *et al.*[12]. However, as Chance [13] has pointed out, our scheme simply requires that aldehyde-enzyme or alcohol-enzyme complexes break down with rates exceeding the dissociation velocities of DPNH·ADH (k_2) and DPN·ADH (k_3), respectively, and therefore are not revealed as ratelimiting steps. Our constants k_4 and k_6 may thus very well be complicated functions of several intermediate steps without this circumstance interfering with the validity of our results. The accuracy of these results is scarcely high enough to entirely rule out *some* rate-limiting influence of enzyme-substrate compounds; but they can certainly not have large effects on the over-all kinetics of the system. The reaction mechanism for liver-ADH seems to be quite different from yeast ADH [14,15]. Some of our experiments also indicate the same.

The binding sites of ADH for DPN and DPNH

Vallee [16] recently found that yeast ADH contains 4 atoms of zinc per molecule. This interesting finding prompted us (in cooperation with Dr. W. Rutter) to analyze liver ADH for metal content. It was found to contain 2 atoms of Zn per molecule, thus one for each of the two DPN(H) molecules that can be bound to one molecule of liver-ADH. There is as yet no conclusive evidence to show that zinc is essential for the activity of ADH, but it appears likely.

As in the case of the O.Y.E. [5] the shape of the pH dependence curves for the "on" (k_1 and k_5) and "off" (k_2 and k_3) velocity constants may help to reveal the chemical nature of the binding sites. In this connection it is important to remember that changes in k_2 and k_3 are entirely connected with changes in the DPNH·ADH respectively DPN.ADH *complexes*, whereas changes in k_1 or k_5 derive from changes either in the *free* coenzymes, or *free* ADH.

Let us first consider the dissociation constants of the titrable groups in DPN and DPNH. They both contain two primary phosphoryl groups with low pK':s, certainly far below the lowest pH value, 5,3, used in our experiments. The pyridinium ion in DPN is a strong base, but the tertiary nitrogen atom in DPNH is a very weak base with pK' below 4 to 5 [17]. Therefore, within our pH range (5.3 to 10) DPN is carrying a positive charge in the pyridine ring which is absent in DPNH.

The pK' of the primary amino groups of the adenine moiety may not have been accurately determined; however, it is expected to be below 5 (from the fact that pK' for adenine is 4.15) and thus carrying no charge in our pH region. Furthermore, it is known that desamino DPN reacts as rapidly with ADH as does DPN [18]. It is thus likely that the $-NH_2$ group in adenine is unimportant for the coupling of DPN(H) to ADH, although we cannot exclude the possibility that —OH (in desamino —DPN(H)) is bound to the same site in ADH, as is $-NH_2$ (in DPN(H)) and thus is able to replace its function. Summarizing, there appears to be no titrable groups in DPN(H) between pH 5.3 and 9—10. Therefore, we need not to consider the possibility that changes with pH in k_1 or k_5 should depend upon the coenzyme. The extra plus charge in DPN^+ compared with DPNH is, however, important.

The titrable groups in the ADH are probably of the same general nature as in any protein, but in addition we have to take into account the presence of

the zinc atoms that are likely to carry a higher positive charge in acidic than in alkaline solutions.

1. As seen from Fig. 8, both k_1 and k_5 have the same general shape as in the case of the "old yellow enzyme", the "on" velocities being high around neutral reaction, decreasing towards both sides on the pH-scale. As in the case of O.Y.E. the decrease towards the alkaline side might depend upon discharge of primary amino groups, which serve in the binding of the pyrophosphate group in DPN(H). The decrease in k_1 and k_5 towards the acidic side cannot have the same cause as assumed in the O.Y.E., where the secondary phosphate group of FMN is titrated around pH 6, since DPN(H) has no such groups. We have so far no explanation to give.

2. The fact that k_1 is 10—100 times higher than k_5 could be connected with the fact that DPNH has two negative charges, DPN a net sum of only one. Repulsion between the positively charged pyridinium ring in DPN and Zn^{++} could cause the difference between k_1 and k_5. This explanation fits with the fact that the positive charge of Zn is higher in acid solution, where the difference between k_1 and k_5 is greater than in neutral or alkaline solution.

3. The pH-dependence of k_3 is most interesting. DPN · ADH shows the highest dissociation velocity around pH 7, decreasing towards both sides of the pH-scale. This is opposite to what was observed in the O.Y.E.[5]. It may be pointed out that a high dissociation velocity at physiological pH is favorable for the function of an enzyme system with "mobile" coenzyme (ADH), whereas the opposite is true for an enzyme with immobile coenzyme (O.Y.E.).

We think, as before [1], that the increased stability of the DPN . ADH complex towards the alkaline side depends upon an acidic group with $pK' = 7.8$, perhaps a sulfhydryl group, in the vicinity of the pyridinium ring. This effect would be inoperable in the DPNH · ADH complex, where the pyridine ring is uncharged; in accordance herewith k_2 remains constant in the alkaline range. The parallel decrease of k_2 and k_3 towards the acidic range could be caused by imidazole groups near the pyrophosphate groups of DPN(H) taking on positive charge in acid solution ($pK' = 6.4$).

4. The possibility should be pointed out that zinc, which is firmly bound to the ADH, probably by several of its possible six covalent bonds, could use one or two of the residual ones to bind and activate the substrates.

Anion effects

The effects of chloride and formate have so far been studied only at pH 7.15. Therefore a detailed discussion on the conclusions to be drawn from their influence on the ADH-system has to await further data. However, it is seen from Table 3 and Fig. 3 that both formate and chloride decrease k_3, which means a stabilization of the DPN · ADH complex; and both cause a decrease in k_6, which means that they inhibit the reaction of DPN · ADH with alcohol. In addition, chloride decreases k_1 and k_5, and increases k_2. As seen from Fig. 3 this leads to the remarkable effect that chloride stimulates the reaction velocity in a system with high concentrations of both DPN and alcohol, but inhibits when either of them is low.

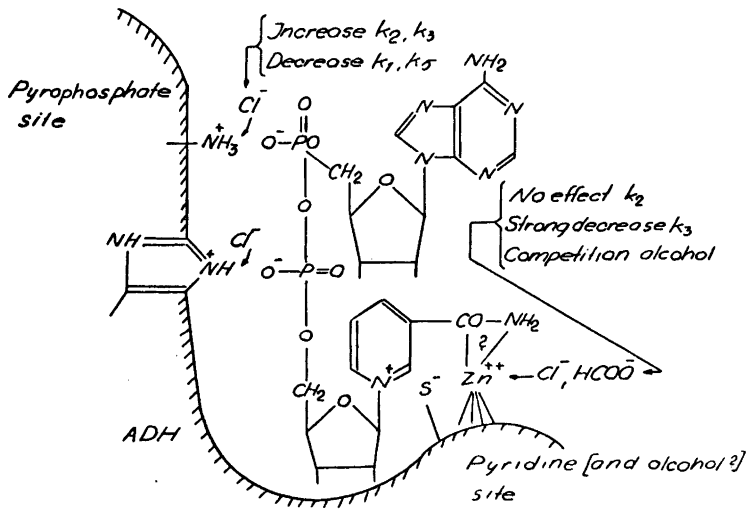
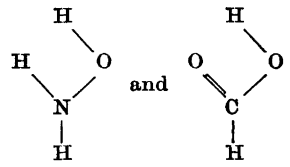


Fig. 11. Tentative scheme for anion effects on the DPN · ADH complex.

The following is a working hypothesis for the explanation of the anion effects: The anions interact with two different binding sites for DPN, the pyridine site and the pyrophosphate site. From our experience with O.Y.E.[5] we would expect chloride to increase k_2 and k_3 and perhaps decrease k_1 and k_5 at the pyrophosphate site (depending upon pH). This is what happened except that k_3 was instead decreased by chloride. Formate likewise decreased k_3, but had no effect on k_1, k_2 or k_5. It appears reasonable to assume that the effect on k_3 of both formate and chloride is at the pyridine site, where the different behaviour of DPN and DPNH is explained by the positive charge which is present in DPN and not in DPNH. Anions could increase the stability of this binding by attaching themselves to a positive group, for example zinc, thereby releasing its repellant effect on the pyridinium ring. This effect would be absent in DPNH · ADH. If the positive group (Zn ?) served as binding site for alcohol, the competition between anions and alcohol could be understandable. The effects of chloride and formate as explained above, are pictured in Fig. 11. It should be unnecessary to emphasize that the illustration is highly speculative.

The fact that formate acts at least 10 times stronger than chloride on k_3 and k_6 indicates that formate fits especially well into the pyridine binding site. This could perhaps be related to the effect of hydroxylamine, studied by Kaplan and his associates [11]. Hydroxylamine, just as formate, has a strongly inhibitory effect on the oxidation of ethanol by DPN · ADH; they both compete with the alcohol; and they both increase the stability of the DPN · ADH linkage. The similar effects are understandable in view of the resemblance of the formic acid and the hydroxylamine molecules



Kaplan *et al.*[11] found that the DPN · NHOH · ADH complex has an absorption band at 300 mμ of about the same height as the DPNH-band at 340 mμ. We therefore investigated whether a similar band could be traced in solutions of DPN and ADH (equimolar amounts) with increasing amounts of formate, at pH 7. No extra bands were found in the ultraviolet, not even with high concentrations of formate. Hydroxylamine and formate are thus different in this respect.

The opposite effects of chloride on k_2 and k_3, and the decreasing effect of formate on k_3 without change in k_2, lead to a change in D_{ox}/D_{red}, as seen from Table 4. This means that the oxidation-reduction potential of the enzyme complex at pH 7.15 will be lowered by 15 mV by 0.15 M chloride and by 17 mV by 0.015 M formate.

The anion effects on the liver-ADH system are in some cases amazingly strong, as seen from Table 6. The results show how extremely carefully the ionic conditions and not only pH have to be considered when studying enzyme-coenzyme reactions.

The effect of anions on the redox potential of the ADH system led us to consider the idea that a salt-sensitive system consisting of an oxido-reducible part reversibly attached to a "carrier" might be operating in certain nerve receptors, transforming a change in ion concentration to a potential change and an electrical discharge. Some recent work by Zotterman *et al.*[18] give some support to this hypothesis. They found particular "water taste" receptors in the cat tongue, that responded with electrical discharges in the corresponding "water fibres" when water, or acetate, or formate solutions were rinsed over the tongue. Salts of strong acids (NaBr, NaI, $NaNO_3$, NaCl, KCl and $CaCl_2$) all abolished this response. It should be recalled that acetate and formate were without effect on the dissociation of the old yellow enzyme, whereas the anions of strong acids all caused increased dissociation, possibly changing the redox potential as in the case of ADH.

Finally, the strong effect of substances like cyanide, hydroxylamine, pyruvoxim and formate on the reaction velocity in the ADH-system is perhaps of considerable interest from a wider point of view. There is no reason to believe that any of these substances, with the possible exception of formate, are present in detectable concentrations in the living cells. But since these chemically very different substances can all react with the pyridine ring, we shall have to look for such effects with substances known to occur physiologically in the cells. This may lead to the discovery of mechanisms for the coordination and regulation of the different enzymatic activities in the cells.

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Graphical Methods for Determining Equilibrium Constants

I. Systems of Mononuclear Complexes

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A new graphical method for computing successive stability constants from experimental values of the degree of formation of the system is described. The method is particularly useful for systems in which more than two complexes coexist. Other methods of calculating stability constants are reviewed critically. The stability constants of the cadmium cyanide system are calculated from Leden's experimental data by a number of the methods, and the advantages of the present treatment over existing methods are discussed.

Numerous experimental studies of equilibria in solution have been made in the last fifteen years, and some of the methods used to calculate the stability constants have been reviewed recently by Sullivan and Hindman [1]. A new method of calculating these constants is outlined in this paper, and its relationship to existing methods is described.

The overall, stoichiometric stability constant of the mononuclear species, ML_n, ($0 \leqslant n \leqslant N$) formed from a central group, M, and n ligands, L, is defined by

$$\beta_n = [ML_n]/[M][L]^n \tag{1}$$

where charges are omitted for clarity. The total, analytical concentrations of the central group, C_M, and of the ligand, C_L are given by

$$C_M = \sum_{n=0}^{n=N} \beta_n[M]\ [L]^n \tag{2}$$

and

$$C_L = [L] + \sum_{n=1}^{n=N} n\beta_n\ [M]\ [L]^n \tag{3}$$

The degree of formation of the system, or ligand number, $\bar{n}$, may be defined as the average number of ligands combined with each central group [2]. Thus

$$\bar{n} = (C_L - [L])/C_M \tag{4}$$

The degree of formation of a given complex, ML_c, is given by

$$\alpha_c = [ML_c]/C_M \tag{5}$$

Then it follows [3] that

$$\bar{n} = \sum_{n=1}^{n=N} n\beta_n [L]^n / \sum_{n=0}^{n=N} \beta_n [L]^n \quad (6)$$

or

$$\sum_{n=0}^{n=N} (\bar{n}-n)\beta_n [L]^n = 0 \quad (6a)$$

and

$$\alpha_c = \beta_c [L]^c / \sum_{n=0}^{n=N} \beta_n [L]^n \quad (7)$$

Whence

$$\frac{d \log \alpha_c}{d p[L]} = (\bar{n}-c) \quad (8)$$

If the activity coefficients of all species are held constant, (*e. g.* by the use of a salt background, in which gross changes in the composition of the ionic medium are avoided, *cf.* Ref.[4]), the terms β_n refer to stoichiometric stability constants. In any experiment, the total concentrations, C_M and C_L, of metal and ligand will be known and, in principle, the determination of at least N values of the concentration of one of the species is sufficient for the computation of the stability constants. Methods for obtaining values of the stability constants from sets of data [M], [L]; α_c, [L]; and $\bar{n}$, [L] are discussed below.

In order to test for the possible presence of polynuclear complexes, measurements should be obtained at a number of values of C_M. If $\bar{n}$ or α_c is a function of both [L] and C_M, at least one polynuclear complex, M_pL_q ($p > 1$), is present, and the stability constants cannot be obtained by the methods described in this paper, unless the data can be extrapolated to low concentrations of metal at which polynuclear species can be neglected [5,6]. Other methods for computing stability constants of mononuclear complexes from data obtained in the presence of polynuclear complexes are treated elsewhere [7,8]. If no polynuclear complexes are formed, $\bar{n}$ and α_c are functions of [L] only, and are independent of C_M (*cf.* equations 6 and 7). In this case, the values of the stability constants β_n, may conveniently be calculated from the data $\bar{n}$, [L] as follows.

PROPOSED GRAPHICAL TREATMENT OF THE DATA $\bar{n}$, [L]

As C_M and C_L are known, the value of $\bar{n}$ can be calculated from equation (4) if the concentration of free ligand, [L], is measured experimentally. Rearrangement of equation (6a) gives

$$\frac{\bar{n}}{(1-\bar{n})[L]} = \beta_1 + \beta_2 \frac{2-\bar{n}}{1-\bar{n}} [L] + \sum_{n=3}^{n=N} \frac{n-\bar{n}}{1-\bar{n}} \beta_n [L]^{n-1} \quad (9a)$$

Thus the plot of $\bar{n}/(1-\bar{n})[L]$ against $(2-\bar{n})[L]/(1-\bar{n})$ tends to a straight line of intercept β_1 and of slope β_2, as $[L] \to 0$. An accurate value of any constant, β_t, may be obtained by using a generalisation of equation (9a). Dividing equation (6a) by $(t - \bar{n})[L]$ (where $0 < t < N$), and rearranging

$$\sum_{n=0}^{n=t-1} \left(\frac{\bar{n}-n}{t-\bar{n}}\right) \beta_n [L]^{n-t} = \beta_t + \sum_{n=t+1}^{n=N} \left(\frac{n-\bar{n}}{t-\bar{n}}\right) \beta_n [L]^{n-t} \quad (9)$$

If values of $\beta_1, \beta_2, \ldots \beta_{t-1}$, have previously been calculated, the left-hand side of equation (9) is known. A plot of this term against $(t+1-\bar{n})[L]/(t-\bar{n})$ then gives β_t as the intercept (and an approximate value of β_{t+1} as the limiting slope as $[L] \to 0$).

When $t = N-1$, equation (9) becomes

$$\sum_{n=0}^{n=N-2} \left(\frac{\bar{n}-n}{N-1-\bar{n}}\right) \beta_n [L]^{n-N+1} = \beta_{N-1} + \beta_N \left(\frac{n-\bar{n}}{N-1-\bar{n}}\right) [L] \qquad (9b)$$

so that a plot of the left-hand side of equation (9b) against $(n-\bar{n})[L]/(N-1-\bar{n})$ will be a straight line of intercept β_{N-1} and of slope β_N. Now any errors in the values of $\beta_1 \ldots \beta_{t-1}$ will accumulate in the value of β_t. An alternative rearrangement of equation (6a) gives

$$\left(\frac{N-\bar{n}}{N-1-\bar{n}}\right) [L] = \frac{\beta_{N-1}}{\beta_N} + \frac{\beta_{N-2}}{\beta_N} \left(\frac{\bar{n}-N+2}{N-1-\bar{n}}\right) [L]^{-1} + \sum_{n=0}^{n=N-3} \left(\frac{\bar{n}-n}{N-1-\bar{n}}\right) \frac{\beta_n}{\beta_N} [L]^{n-N+1} \qquad (10)$$

Thus the plot of $(N-\bar{n})\,[L]/(N-1-\bar{n})$ against $(\bar{n}-N+2)/(N-1-\bar{n})\,[L]$ will have an intercept of β_{N-1}/β_N, and a limiting slope of β_{N-2}/β_N, as $[L]^{-1} \to 0$. All the ratios β_t/β_N (where $\beta_0/\beta_N = 1/\beta_N$) may be found in an analogous way, and equation (10) may be used to check the values of the stability constants obtained from equation (9).

The application of this method to the determination of the stability constants of the cadmium cyanide system, and its advantages over other methods, are discussed below. (See p. 1174 and Figs. 1a, 1b and 1c).

Special cases of the present treatment

(a) Only one complex formed.

If $N = 1$, equation (9) reduces to

$$\bar{n}/(1-\bar{n}) = \beta_1[L] \qquad (11)$$

or

$$\log\ \bar{n}/(1-\bar{n}) = \log\ \beta_1 + \log\ [L] \qquad (11a)$$

Equation (11a) is familiar as Henderson's or Hasselbalch's equation [10,11], and has been widely used for the determination of the dissociation constants of monobasic acids. It is also useful for determining constants from $\bar{n}$, [L] data obtained over a very limited range, *e. g.* if very strong or very weak complexes are formed, or if precipitation occurs at low values of $\bar{n}$.

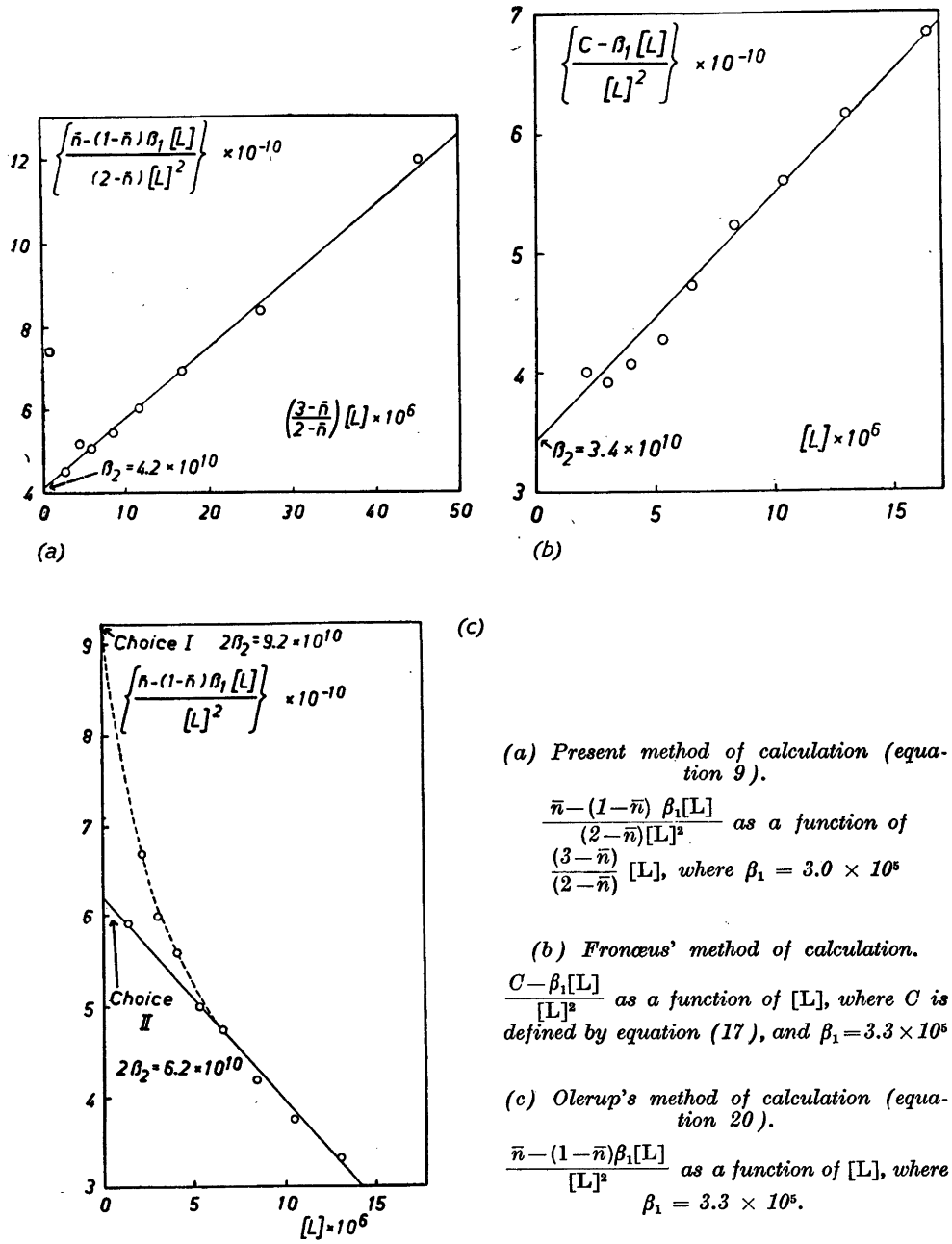
(b) Only two complexes formed.

When $N = 2$, $t = 1$, and equation (9) reduces to the linear form derived previously [13]

$$\frac{\bar{n}}{(1-\bar{n})\,[L]} = \beta_1 + \beta_2 \frac{(2-\bar{n})\,[L]}{(1-\bar{n})} \qquad (12)$$

Alternative rearrangement gives an equation which is equivalent to that used earlier by Speakman [14] for the determination of the dissociation constants of dibasic acids.

$$\frac{1}{\beta_2} + \frac{(\bar{n}-1)\,[L]}{\bar{n}} \frac{\beta_1}{\beta_2} = \frac{(2-\bar{n})\,[L]^2}{\bar{n}} \qquad (12a)$$



(a) Present method of calculation (equation 9).

$\frac{\bar{n}-(1-\bar{n})\ \beta_1[L]}{(2-\bar{n})[L]^2}$ *as a function of* $\frac{(3-\bar{n})}{(2-\bar{n})}$ [L], *where* $\beta_1 = 3.0 \times 10^5$

(b) Fronæus' method of calculation.

$\frac{C-\beta_1[L]}{[L]^2}$ *as a function of* [L], *where C is defined by equation (17), and* $\beta_1 = 3.3\times 10^5$

(c) Olerup's method of calculation (equation 20).

$\frac{\bar{n}-(1-\bar{n})\beta_1[L]}{[L]^2}$ *as a function of* [L], *where* $\beta_1 = 3.3 \times 10^5$.

Fig. 1. Determination of β_2 for the cadmium cyanide system by extrapolation to zero concentration of free cyanide ion.

OTHER GRAPHICAL METHODS FOR COMPUTING STABILITY CONSTANTS

A number of other authors have given methods for obtaining stability constants by extrapolation of certain functions to zero free ligand concentration. In each case, a polynomial in [L] or $[L]^{-1}$, the coefficients of which are the required β_n, is set up using the experimental data. The graphical extrapolations reduce the polynomial to N linear equations. This general method has also been used to obtain specific rate constants for an exchange reaction [9].

I. Data [M], [L]

If the concentration of free metal, [M], is determined, the value of [L] may be obtained from the relationship

$$C_L \sim [L] \tag{13}$$

and by successive approximations [3,16]. Alternatively, Hedström has shown [7] that

$$\log \frac{[L]}{C_L} = \left[\int_0^{C_M} \left(\frac{\partial \log [M]/C_M}{\partial C_L} \right)_{C_M} dC_M \right]_{C_L} \tag{13a}$$

The data [M], [L] may be expressed as a simple polynomial in [L], for which the coefficients are the stability constants. Thus Leden's function [15,16]

$$A = (C_M - [M])/[M][L] = \beta_1 + \beta_2[L] \ldots = \sum_{n=1}^{n=N} \beta_n [L]^{n-1} = \frac{g}{[L]} \tag{14a}$$

where g is the summation, $\sum_{n=1}^{n=N} \beta_n [L]^n$. The value of β_1 is obtained as the intercept of the plot of A against [L]. Similarly, the plot of $(A-\beta_1)/[L]$ against [L] gives an intercept of β_2. In general,

$$F_i = \frac{A - \sum_{n=1}^{n=i-1} \beta_n [L]^{n-1}}{[L]^{i-1}} = \beta_i + \sum_{n=i+1}^{n=N} \beta_n [L]^{n-i} \tag{14}$$

so that, if values of $\beta_1 \ldots . \beta_{i-1}$ have been calculated, the term F_i is known. β_i may then be obtained as the intercept of the plot of F_i against [L].

Olerup [17] defines a function

$$B = C_M/[M] = 1 + \beta_1 [L] \ldots = \sum_{n=0}^{n=N} \beta_n [L]^n = 1 + g \tag{15}$$

Thus Olerup's method of obtaining β_1 as the intercept of the plot of $(B-1)/[L]$ against [L] is identical to that of Leden.

If required, values of $\bar{n}$ can be calculated from the data [M], [L] (see below), but Leden's treatment clearly provides a more direct method for computing stability constants from the experimental measurements.

II. Data $[ML_c]$, [L]

In cases where the concentration of one of the complexes, $[ML_c]$, is measured, values of [L] may again have to be obtained by successive approximations. Values of α_c may be calculated using equation (5). The ligand number, $\bar{n}$, may

then be obtained as the slope of the curve log α_c, (p[L]) (see equation (8)), and used to calculate the stability constants by one of the methods described in this paper.

Alternatively, rearrangement of equation (7) gives

$$\frac{[L]^c}{\alpha_c} = \frac{1}{\beta_c} + \frac{\beta_1}{\beta_c}[L] \ldots = \sum_{n=0}^{n=N} \frac{\beta_n}{\beta_c}[L]^n = \frac{1+g}{\beta_c} \qquad (16)$$

so that the value of $1/\beta_c$ may be obtained as the intercept of the plot of $[L]^c/\alpha_c$ against [L], and values of the ratios β_1/β_c, β_2/β_c ..... may be obtained by successive extrapolations. A modified form of equation (16) has been used to compute stability constants from partition data [18], from which the product of α_c and a partition coefficient is obtained. When the concentration of free metal is measured, $\alpha_c = \alpha_0$, and equation (16) reduces to (15).

III. Data, $\bar{n}$, [L]

When [L] is measured, $\bar{n}$ may be calculated directly from equation (4).

(a) Fronæus' Method. The value of the summation, g may be obtained from the experimental data by graphical integration. Thus, Fronæus' function, C, is given by [5,6]

$$C = \exp.\int_0^{[L]} \frac{\bar{n}}{[L]}\, d[L] = \exp.\int_0^{[L]} \bar{n}\ d\ \ln\ [L] = \sum_{n=0}^{n=N} \beta_n [L]^n = 1 + g \qquad (17)$$

The value of the residual integral may be estimated if it is assumed that only the first complex, ML, exists in appreciable concentrations at very low values of [L]. Then

$$\lim_{[L]\to 0} \int_0^{[L]} \frac{\bar{n}}{[L]}\, d\ [L] = \frac{1}{(1-\bar{n})} \qquad (18)$$

As the function C is identical to that obtained by Olerup [17] (B, equation (15)) from measurements of the concentration of free metal, values of the constants $\beta_1 \ldots \beta_N$ may be obtained by successive extrapolation, as described above. The constants may also be calculated in an analogous way using the function

$$\frac{g}{[L]^N} = \beta_N + \beta_{N-1}[L]^{-1} \ldots = \sum_{n=1}^{n=N} \beta_n [L]^{n-N} \qquad (19)$$

which is a polynomial in $[L]^{-1}$. It is advisable to use equation (19) to check the values of the higher constants which, when calculated from equation (17) reflect any errors in the lower constants. Fronæus' method may also be used to calculate the concentration of free metal ions from measurements of the free ligand concentration, *cf.* eqns. (15) and (17).

(b) Olerup's Method. Equation (6a) may be rearranged [17,19] to

$$D = \frac{\bar{n}}{[L]} = (1-\bar{n})\beta_1 + (2-\bar{n})\beta_2[L] \ldots = \sum_{n=1}^{n=N} (n-\bar{n})\beta_n [L]^{n-1} \qquad (20a)$$

and β_1 obtained from the intercept of the plot D, ([L]). Similarly, the term $2\beta_2$ may be obtained as the intercept of the plot of $D-(1-\bar{n})\ \beta_1/[\mathrm{L}]$ against [L]. In general

$$F_t' = \frac{D - \sum_{n=1}^{n=t-1} (n-\bar{n})\ \beta_n\ [\mathrm{L}]^{n-1}}{[\mathrm{L}]^{t-1}}$$

$$= (t-\bar{n})\ \beta_t + \sum_{n=t+1}^{n=N} (n-\bar{n})\ \beta_n\ [\mathrm{L}]^{n-t} \qquad (20)$$

If values of $\beta_1 \ldots \beta_{t-1}$ have been calculated, the term F_t' is known, and $t\ \beta_t$ may, in principle, be found as the intercept of the plot F_t', ([L]). Unfortunately, since the coefficient of the required β_t is a variable, $(t-\bar{n})$, the plots tend to be markedly curved, even at low values of [L], and accurate extrapolation to zero free ligand concentration may be difficult (see Fig. 1c). The method has, however, been successfully used to determine the first one or two constants for systems in which weak complex formation occurs [17,19,20].

(c) The present method. The treatment described on p. 1167 has the advantage of avoiding the graphical integration which is necessary in Fronæus' method; thus no preliminary smoothing of the data is required. Compared with Olerup's method, the treatment has the further advantage that the coefficient of β_t is a constant (*cf.* equation (9)). The functions to be extrapolated are therefore much less curved, and more accurate values of the stability constants may be obtained. In common with other methods for calculating accurate values of stability constants, the present method is somewhat laborious, and requires a large number of experimental points, well spaced over the range $0 < \bar{n} < N$, and preferably of high precision.

OTHER METHODS FOR COMPUTING STABILITY CONSTANTS

It has been seen that polynomial equations in [L], of the type (9), (14) and (20), can be solved for values of the stability constants by several closely-related limiting graphical methods, which reduce the polynomial to N linear functions. A number of other methods, which have also been used for computing stability constants, will be discussed.

(a) Solution of simultaneous equations. Some workers have obtained values of $\beta_1 \ldots \beta_N$ by solving N simultaneous equations of the type (6a) [18,21,27]. Ideally, if $m > N$ corresponding values of $\bar{n}$ and [L], or [M] and [L], were available, $m!/N!\ (m-N)!$ sets of equations should be solved in order to use all the data, and the computation would be very tedious. The equations would probably be inconsistent on account of experimental errors and equations formed from neighbouring points might be ill-conditioned. The problem of choosing the 'best' set of constants would then arise [13,27].

(b) Successive approximations. Stability constants are often obtained from equation (6a) by successive approximations. J. Bjerrum [3] transformed (6a) to an equation of the type

$$\frac{\beta_t}{\beta_{t+1}} = \frac{1}{[\mathrm{L}]_{\bar{n}=t-\frac{1}{2}}} \cdot \frac{\sum_{n=0}^{n=t-1} (1+2n)\ [\mathrm{L}]^{-n} \beta_n^{-1}}{\sum_{n=0}^{n=N-t} (1+2n)\ [\mathrm{L}]^{n} \beta_{n+t} \beta_t^{-1}} \qquad (21)$$

or

$$\frac{\beta_t}{\beta_{t+1}} \sim \frac{1}{[\mathrm{L}]_{\bar{n}=t-\frac{1}{2}}} \qquad (21a)$$

where t is again an integer such that $0 < t < N$. Approximate values of the ratios β_t/β_{t+1} may be obtained from equation (21a), and refined by successive approximations, using equation (21). If two or more species in a system of complexes are of comparable stability, this method becomes very laborious (*cf.* Ref.[22]). A further disadvantage is that only N sets of experimental data $\bar{n}$, [L] are used. The use of simplifications of equations (21) and (21a) for the special case where $N = 2$ has been discussed elsewhere [13].

Scatchard [23] has recently combined features of Bjerrum's treatment and of the extrapolation method in an attempt to reduce the number of successive approximations required. As [L] → 0 or ∞, the function

$$Q = \frac{\bar{n}}{(N-\bar{n})\ [\mathrm{L}]}$$

has limiting values of β_1/N and $N\beta_N/\beta_{N-1}$, respectively. Moreover, approximate values of the ratios β_2/β_1, and β_{N-1}/β_{N-2} may be obtained from the limiting values of d ln Q/ d $\bar{n}$, as $\bar{n} \to 0$ and N. These values are then refined by successive approximations.

(c) Curve-fitting. Values of the stability constants may also be obtained by comparing experimental formation curves, $\bar{n}$, (p[L]), with curves calculated from equation (6), using different values of β_n. This approach, which will be discussed elsewhere by Sillén [24] is particularly useful for systems in which only one complex is formed ($N=1$) and the shape of the formation curve is unique. The value of β_1 is then obtained from the position of the curve on the p[L] axis, using equation (11a). The method has previously been successfully applied to the determination of dissociation constants of organic acids [25].

If two complexes are formed, the position of the curve is determined by the value of β_2, while the shape depends on the ratio β_1^2/β_2. Accurate evaluation of stability constants for systems of this type would therefore require formation curves calculated for a large number of values of this ratio. The difficulty may be overcome by using a set of theoretical curves in which some property, P, of the formation curve (*e.g.* $P =$ d $\bar{n}$/d p[L] or $(\bar{n}_1-\bar{n}_2)/(\mathrm{p[L]}_1-\mathrm{p[L]}_2)$) is plotted against a function of β_1^2/β_2 for several values of $\bar{n}$, *cf.* Ref.[13]. Values of β_1^2/β_2, corresponding to the experimental values of P, may then be obtained from the appropriate theoretical curves, and the mean taken. The value of β_1 is obtained by combining this value with the value of β_2 obtained from the position of the curve on the p[L] axis.

On account of the very large number of curves that would be required, it is impracticable to obtain accurate values of the stability constants if more than two complexes are formed, unless the constants are very widely separated. Approximate values may, however, be calculated if certain assumptions are made about the term $R_n=\beta_n^2/\beta_{n-1}\ \beta_{n+1}$. (Here R_n is the ratio K_n/K_{n+1}, where $K_n = [ML_n]/[ML_{n-1}][L]$). J. Bjerrum [3] proposed a method assuming that the addition of ligands was statistical, *i. e.* that $R_n = f(n, N)$. Dyrssen and Sillén [26] assumed that R_n is a constant, and described systems in terms of two parameters

$$a = \frac{1}{N} \log\ \beta_N$$

and

$$b = \log\ \beta_n - \tfrac{1}{2} \log\ \beta_{n-1}\beta_{n+1} = \tfrac{1}{2}\ (\log\ K_n - \log\ K_{n+1})$$

The value of b may be obtained by comparing the shape of the experimental log a_c, (p[L]) curve with theoretical curves calculated for given values of b, and that of a found from the position of the curve on the p[L] axis. This method is particularly useful for obtaining approximate constants from partition equilibria, for which data of the highest accuracy cannot be obtained, but the treatment is only exact if not more than two complexes are formed. Further examples of this type of approach will be discussed elsewhere [28].

CALCULATION OF THE STABILITY CONSTANTS FOR THE CADMIUM CYANIDE SYSTEM

As an illustration of the present method, and of some of the methods reviewed above, stability constants were calculated from Leden's data for the cadmium cyanide system [16]. Leden measured the concentration of free cadmium, and calculated values of $\bar{n}$ by means of equation (8). The stability constants obtained are given in Table 1.

Table 1. Stability constants of the cadmium cyanide system in a 3 M sodium perchlorate medium at 25° C.

Data	Method	$10^{-5}\beta_1$	$10^{-10}\beta_2$	$10^{-15}\beta_3$	$10^{-18}\beta_4$	Ref.
[M], [L]	Leden	3.0	4.0	1.6	6.0	Leden [16]
	Dyrssen & Sillén	~3.2	~3.2	~1.0	~10	This work
$\bar{n}$, [L]	Bjerrum	3.5	4.0	1.8	7.0	Leden [16]
	Olerup: choice I	3.3	4.6	1.1	40	This work
	» II		3.1	2.7	1.3	» »
	Fronæus	3.3	3.4	1.8	5.8	» »
	Present method	3.0	4.2	1.5	5.7	» »

Some examples of the graphical extrapolations are shown in Fig. 1. The present method (Fig. 1a) and that of Fronæus (Fig. 1b) give good linear extrapolations, but the curved function (Fig. 1c) given by Olerup's method was so difficult to extrapolate that two possible values of β_2 are given. Moreover, the extrapolation of F_3' (equation (20)), following choice II throws most weight on points in the region $\bar{n} < 1.9$. Similarly, the value of β_4, following choice I, is obtained mainly from data corresponding to $\bar{n} < 2.24$. It appears to be necessary to select arbitrarily suitable ranges of values of $\bar{n}$ for each extrapolation in order to use Olerups method effectively, *cf.* Ref.[20].

The validity of the sets of constants calculated by the different methods was tested by substitution of these values into equation (6). Values of $\bar{n}_{calc.}$ corresponding to given values of [L] were then calculated and compared with the appropriate experimental values, $\bar{n}_{expt.}$. Representative values of

$$\Delta\bar{n} = \bar{n}_{expt.} - \bar{n}_{calc.}$$

are given in Table 2.

Table 2. Comparison of experimental and calculated formation functions for the cadmium cyanide system.

p[L]	5.857	5.519	5.077	4.782	4.265	3.907	3.248
$\bar{n}_{expt.}$	0.393	0.778	1.52	2.06	2.75	3.08	3.68
Method				Δn			
Leden [a]	−0.003	−0.009	0.01	0.00	0.03	0.06	−0.04
Dyrssen & Sillén	0.014	0.064	0.13	0.12	−0.13	−0.29	−0.15
Olerup, choice I	0.023	0.016	0.00	0.007	0.51	0.62	0.26
» II	0.001	−0.017	0.06	0.12	0.04	−0.11	−0.49
Fronæus	0.002	−0.026	−0.07	0.00	0.05	0.06	−0.06
Present method	0.000	−0.004	0.00	−0.04	0.00	0.04	−0.02

[a] The value of Δn has been calculated from Leden's preferred set of constants, obtained by combination of the values calculated by Leden's and Bjerrum's methods, and given in Table 1.

It is seen that the present method, and those of Leden and Fronæus, yield values of the stability constants in very good agreement with the experimental data. Much larger discrepancies are found with Olerup's method, doubtless on account of the difficulty in extrapolation. The constants obtained by means of Dyrssen and Sillén's 'two parameter' method cannot be expected to agree well with the experimental data, as the ratio K_n/K_{n+1} is not a constant for the cadmium cyanide system, although $K_1/K_2 \sim K_2/K_3$.

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Studies on the Hydrolysis of Metal Ions

XII. The Hydrolysis of the Vanadium(IV) ion

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Spectrophotometric and potentiometric measurements made in a 3 M perchlorate medium at 25° C indicate that vanadium(IV) exists as the vanadyl ion, VO^{2+} (or $V(OH)_2^{2+}$), in the range of acidity 3 M $>[H^+]>$ 0.002 M. Potentiometric data for the hydrolysis of the vanadyl ion, for total concentrations of vanadium in the range 0.05 M $\gg$ M $\gg$ 0.005 M, may be explained by assuming that only two further complexes are formed in solution. For convenience these will be written as $VO.OH^+$ and $(VO)_2(OH)_2^{2+}$, although they may be combined with water molecules, or sodium or perchlorate ions. The following values of the equilibrium constants were obtained:

$\varkappa_{1,1} = [VO.OH^+]\,[H^+] / [VO^{2+}] = 10^{-6.0 \pm 0.1}$

$\varkappa_{2,2} = [(VO)_2(OH)_2^{2+}]\,[H^+]^2/[VO^{2+}]^2 = 10^{-6.88 \pm 0.04}$

Precipitation occurred when approximately 10 % of the vanadyl ions were further hydrolysed.

Little quantitative work on the hydrolysis of tetravalent vanadium has been reported. Britton and Welford [1] (*cf.* Ref.[2]) have measured the changes in pH which occur when a solution of vanadyl sulphate, $VOSO_4$, is titrated with alkali, but as both the total concentration of vanadium, and the ionic strength, varied throughout the titration, no detailed interpretation of these data is possible. Their results do, however, indicate that, in strongly acidic solutions, vanadium(IV) exists as the "vanadyl" ion, VO^{2+}. A precipitate of composition $VO(OH)_2$ is formed on addition of alkali. It is somewhat soluble in excess of alkali to give "vanadites", which are readily oxidised by air.

Jones and Ray [3] measured the pH of solutions of pure vanadyl sulphate of concentrations 0.5 M to 0.0001 M. Their data have subsequently been used to calculate the equilibrium constant, $\varkappa_{1,1} = 4.4 \cdot 10^{-6}$ for the reaction

$$VO^{2+} + H_2O \rightleftharpoons VO(OH)^+ + H^+$$

at infinite dilution [4]. It was assumed that $VO(OH)^+$ was the only hydroxyl complex formed, and that the presence of HSO_4^- ions and vanadium sulphate complexes could be neglected.

The influence of the hydrogen ion concentration on the formal redox potential V(IV)/V(V) has been investigated by several authors (Ref.[5] and refs. therein,[6]). The data obtained in strongly acidic solution indicate that vanadium (IV) exists as the vanadyl ion, VO^{2+}. Measurements at higher values of pH are difficult to interpret, as hydrolysis products of both valency states are undoubtedly present. As would be expected, the value of the half-wave oxidation potential [7] of vanadium(IV) also varies with pH.

As a large number of metal ions react with water to form polynuclear hydrolysis products, investigation of equilibria in systems of this type requires accurate data at a number of different total concentrations of metal. The hydrolysis of tetravalent vanadium was therefore studied using the techniques previously developed in this laboratory (*cf.* previous papers in this series, *e. g.*[8-15]).

List of symbols

The symbols adopted are mainly those used in the previous papers in this series.

A_ε optical absorbancy
c_n concentration of complex $VO((OH)_tVO)_n$
E measured potential in mV
E' $= E - 59.16 \log h$ (1)
g^* $= k_n u^n$ (11)
H total analytical concentration of hydrogen ions in the system, assuming that all vanadium is in the form VO^{2+} (4)
H' total analytical concentration of hydrogen ions in the system, assuming that all vanadium is in the form V^{4+} (3)
h concentration of free hydrogen ions
k equilibrium constant, defined by $k = k_{n+1}k_n^{-1}$ (hypothesis IIIa)
k_0 $= k_n k^{-n}$ (hypothesis IIIa)
k_n = equilibrium constant for the reaction $(n+1)VO^{2+} + tn\ H_2O = VO((OH)_t)_n^{2+} + tn\ H^+$
K_w ionic product of water $= [H^+]\ [OH^-]$
M total concentration of vanadium
M mole/l
m concentration of free vanadyl ions, VO^{2+}
N number of $(OH)_tVO$ links in a unique complex
n variable number of $(OH)_tVO$ links in the complex $VO((OH)_tVO)_n$
$\bar{n}$ average number of links per complex
p number of hydroxyl groups in complex $(VO)_q(OH)_p$
q number of vanadyl groups in complex $(VO)_q(OH)_p$
s, s' hydration numbers
t number of hydroxyl group per vanadyl group in link
u $= mh^{-t}$ (10)
x $= \log M - t \log h$ (7)
y $= Z/t$ (6)
Z average number of hydroxyl groups per vanadyl group $= (H-h)/M$ (2)
$\varkappa_{p,q}$ equilibrium constant for the reaction $q\ VO^{2+} + p\ H_2O \rightleftharpoons (VO)_q(OH)_p + p\ H^+$

All species may be combined with the medium ions, Na^+ and ClO_4^-, or with molecules of the solvent; thus it is impossible to distinguish between two hydroxyl groups, and one oxo-group, $-O-$.

Concentrations and equilibrium constants will be expressed in M (mole/l) throughout.

METHOD

The main investigation consisted of a series of potentiometric titrations at 25° C. In each titration, the total concentration of vanadium, M, was kept constant, while the total concentration of acid in the system was varied. All solutions were made 3 M with respect to the perchlorate ion by the addition of sodium perchlorate. Since the concentrations of hydrogen ions and metal ions never exceeded 0.4 % and 1.7 %, respectively, of the concentration of perchlorate ions, it can be assumed that the activity coefficients of all species remain constant [8], and hence that true stoichiometric constants may be calculated from the measurements.

Now as trivalent vanadium is oxidised by the perchlorate ion [20], and as no reliable data on the hydrolysis of pentavalent vanadium in 3 M perchlorate solutions were available, the concentration of free vanadyl ions could not be measured with a redox electrode; neither is another electrode which is reversible to vanadium(IV) known. The titrations were therefore followed only by measurement of the free hydrogen ion concentration, h. A glass electrode was used, in combination with the half-cell

$$\text{RE} \,//\, = \text{Ag} \,/\, 0.01 \text{ M } AgClO_4,\ 2.99 \text{ M } NaClO_4 \,/\, 3 \text{ M } NaClO_4$$

For the cell

$$\text{RE} \,//\, H^+ \,/\, \text{Glass } 25° \text{ C}$$

the relationship between the measured potential, E, and the concentration of free hydrogen ions, is given by

$$E = E_0' + 59.16 \log h \qquad (1)$$

The term E_0' includes the standard potentials of the two electrodes, the asymmetry potential of the glass electrode, and the liquid junction potential. In the range of acidity studied ($h \leqslant 0.01$ M), the value of E_0' was found to be independent of h within the limits of experimental error. The value of E_0' was determined for each titration, either by a preliminary acid-base titration in the absence of metal, or by titration in the presence of metal, in the range $0.01 \text{ M} \gg h \gg 0.002$ M. As no hydrolysis occurs in this region (see below), h is identical with the total analytical hydrogen ion concentration, H, defined by equation (4). For each point, H is known from the composition of the solutions, and E_0' is calculated from the measured value of E, using equation (1).

When E_0' is known, and E is measured, h can be obtained from equation (1). As both H and M are known, the average ligand number of the hydroxy complexes, Z, may be calculated for any point of the titration, using the relationship

$$Z = (h—H) \,/\, M \qquad (2)$$

The data may conveniently be discussed in terms of the variation of Z with $\log h$.

The vanadium(IV) species present in strongly acidic solution were investigated spectrophotometrically.

EXPERIMENTAL DETAILS

Reagents

Vanadium (IV) perchlorate solutions were prepared by adding a solution of saturated ammonium carbonate (*p. a.*) dropwise to an aqueous solution of vanadyl sulphate (Merck, *p. a.*) until there was no further precipitation. The precipitate was centrifuged, washed free from sulphate ions, and dissolved in a slight excess of perchloric acid (*p. a.*). The washing was carried out as quickly as possible to avoid aerial oxidation of the neutral precipitate (*cf.* Ref.[2]). The concentration of vanadium(IV) in the solution was determined by titration with aqueous potassium permanganate, which had been standardised against sodium oxalate (*p. a.*), dried at 120° C. In order to test for the possible presence of any vanadium(V), the solution was then reduced with sulphur dioxide. When the excess of sulphur dioxide had been expelled by passing carbon dioxide through the boiling solution, the solution was again titrated with permanganate. Since the amount of permanganate required after this reduction was the same as that used in the first titration, the stock solution contained only tetravalent vanadium. The total concentration of perchlorate ion was found by passing the solution through a column containing the resin Amberlite IR 120, in the hydrogen form, washing with water, and titrating the eluate to the methyl red end-point with standard sodium hydroxide. Dilute solutions of vanadium (IV) perchlorate for titration were prepared by weight.

Perchloric acid solutions were prepared from perchloric acid (*p. a.*) and standardised against potassium hydrogen carbonate (Merck, *p. a.*) which was free from sodium ions, and had been dried in an atmosphere of carbon dioxide.

Sodium hydrogen carbonate solutions were prepared by weighing out the "AnalaR" solid. The concentrations were checked by potentiometric titration against standard perchloric acid, using Gran's method for calculating the end-point [21].

Silver perchlorate solutions were prepared by adding an excess of silver oxide to a boiling solution of perchloric acid. The solution was filtered, and the concentration of silver determined by Volhard's method.

Sodium perchlorate was prepared by neutralising a solution of perchloric acid with sodium hydrogen carbonate. The solutions were analysed both by using a cation exchange resin (see above), and by evaporating and drying to constant weight at 125° C. Good agreement was found between the two methods. No chloride or chlorate ions could be detected in the product.

Apparatus

The titration cell was of the type described by Forsling, Hietanen, and Sillén [9]. The glass electrode was made by Mr. Erik Blomgren, of Uppsala. The potential of the cell

Glass // H^+ / Quinhydrone / Pt

was found to be constant, ± 0.1 mV, in the range $-1.0 \gg \log h \gg -6.0$, showing that equation (1) is valid. Thus, in the range $h < 0.01$ M, where the term E'_0 is effectively independent of h (see p. 1179), the behaviour of the glass electrode can be described by the Nernst equation. The silver reference electrodes were prepared as recommended by Brown [22] and coated with silver chloride by electrolysis for half an hour in 0.1 M hydrochloric acid with a current of 0.3 milliampere per electrode. Potentials were measured to ± 0.1 mV with a Radiometer PHM3i valve potentiometer.

Solutions were introduced into the titration cell from burettes which had been calibrated by the method of weighing, using 3 M sodium perchlorate. The titration solution was stirred by a stream of nitrogen which had been passed through a 3 M sodium perchlorate solution.

Spectrophotometric measurements were made at 25 ± 1° C with a Beckman DU Spectrophotometer, using matched 1.00 cm Corex cells, and minimum slit widths.

Titration procedure

Titrations were carried out in a paraffin oil thermostat at 25.0 ± 0.1° C. 20.0 ml of a solution, A, containing vanadium(IV) perchlorate (concentration 2 *M*) in perchloric acid (~0.005 M) was added to 20.0 ml of perchloric acid (~0.012 M). This mixture, solu-

tion B, was titrated with equal volumes of sodium hydrogen carbonate (~0.03 M) and of solution A. The total concentration of metal, M, then remained constant throughout the titration. Series of measurements were made with M~0.05 M, ~0.02 M, ~0.01 M and ~0.005 M.

Sodium hydrogen carbonate was used as a base in preference to sodium hydroxide to avoid local precipitation (*cf.* Ref.[10]). Values of h <0.0001 M were not required as vanadium(IV) precipitates in this region. Near the end of one titration, the equilibrium potential was noted, and the stream of nitrogen exchanged for a rapid stream of carbon dioxide. The system was left for an hour, and then nitrogen was bubbled through the solution for one minute. The potential was the same as before, indicating that the measurements are not invalidated by complex formation between vanadium(IV) and carbonate ions.

In the early stages of hydrolysis (*e. g.* Z ~0.05, for M ~0.01 M) a steady potential was reached in 2 to 5 minutes. When slightly hydrolysed solutions were back-titrated with acid, the points $Z(\log h)$ fell on the same curves as points obtained by forward titration with alkali, indicating that equilibria are reversible in this region. In solutions of rather higher pH (*e. g.* Z ~0.08, for M~ 0.01 M), equilibrium was reached more slowly. A steady potential was obtained in about half an hour, and remained constant for at least three hours. As the emf. measurements in this region did not seem to be quite reversible, it is possible that some invisible precipitate was present. When visible precipitation occured, no steady potential could be obtained, and the titration was discontinued.

It is well-known that solutions of tetravalent vanadium are readily oxidised by air [2]. Preliminary work on the ageing of hydrolysed solutions of vanadium(IV) perchlorate in the absence of air shows that the perchlorate ion is slowly reduced at room temperature. The reaction takes place rapidly on boiling. After each titration the metal solution was acidified, and analysed for vanadium(IV) and (V), but no oxidation was found to have occurred during the course of the titrations.

VANADIUM (IV) PERCHLORATE IN ACIDIC SOLUTION

The total analytical hydrogen ion concentration, H', was first calculated assuming that the ion V^{4+} is completely unhydrolysed. Then for the stock metal solution, which contains only vanadium (IV) perchlorate and perchloric acid

$$H' = [ClO_4^-] - 4M \qquad (3)$$

Preliminary titrations based on this assumption indicated that, in the range $0.03\ M \gg h \gg 0.002$ M, two hydroxyl groups are bound to each metal atom, *i. e.* that the species $[V(OH)_2^{2+}]_X$ or $(VO^{2+})_X$ is present.

The effect of higher acidities and metal concentrations on the nature of the species present was investigated by comparing the optical absorbancy, A_s, of different solutions. The ratio A_s/M is shown as a function of wavelength in Fig. 1, and is seen to be independent of M in the range $0.936\ M \gg M \gg 0.023$ M, and of h in the range $2.953\ M \gg h \gg 0.027$ M. Fig. 1 is similar to that obtained by Furman and Garner [23], who showed that the absorption spectrum of vanadium(IV) perchlorate is independent of h in the range $1\ M \gg h \gg 0.02$ M. The slight differences between the present results, and those obtained by Furman and Garner in a 2 M perchlorate medium may be due to the large difference in the concentration of the perchlorate ion. The absorbancies of solutions of iron (III) perchlorate are also influenced by the concentration of perchlorate ions [24].

As the ratio A_s/M is independent of the value of M, the species is probably monomeric. For convenience it will be written as VO^{2+}, although it may be

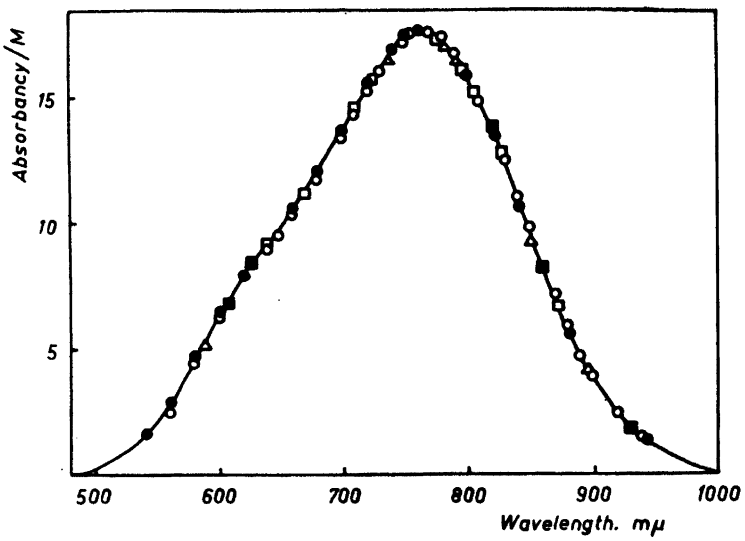

Fig. 1. Visual absorption spectrum of vanadium (IV) perchlorate in acidic solution (3 M perchlorate medium).

	M	*h*
○	0.0234 M	0.0266 M
●	0.0234 M	2.935 M
□	0.0094 M	0.700 M
■	0.0468 M	0.700 M
△	0.0936 M	0.700 M

combined either with water molecules, or with sodium or perchlorate ions [11]. As the ion showed no tendency to combine with protons, even at the highest acidity studied ($h = 2.95$ M), the subsequent results will be discussed in terms of the addition of hydroxyl groups to a central vanadyl ion. H is then defined as the total analytical concentration of hydrogen ions, assuming that all the vanadium is in the form of VO^{2+}. Thus, for the stock solution of vanadium(IV) perchlorate

$$H = [ClO_4^-] - 2M \qquad (4)$$

Similarly, Z is defined as the average number of hydroxyl groups bound to or protons dissociated from, a vanadyl ion. An analogous approach has been used[12] in the study of the uranyl ion, UO_2^{2+}.

The value of H was found using equation (4). For each titration, the total analytical concentration of hydrogen ions in solution A was checked by potentiometric titration with sodium hydrogen carbonate. The titrations were discontinued before any hydrolysis of the vanadyl ion occured (h>0.002 M), and the end-point was found by Gran's method of extrapolation[21]. Similarly, the total analytical concentration of hydrogen ions in solution B was checked by extrapolating the first few points of the actual hydrolysis titration (h>0.002 M) to the end-point. Very satisfactory agreement was found between the interrelated values of the total analytical hydrogen ion concentrations.

THE HYDROLYSIS OF THE VANADYL ION, VO^{2+}.

The data Z (log h)$_M$ were obtained from the potentiometric measurements, using equations (1) and (2). They are shown in Table 1 and plotted in Fig. 2. If only mononuclear complexes were formed, Z would be a function of h only,

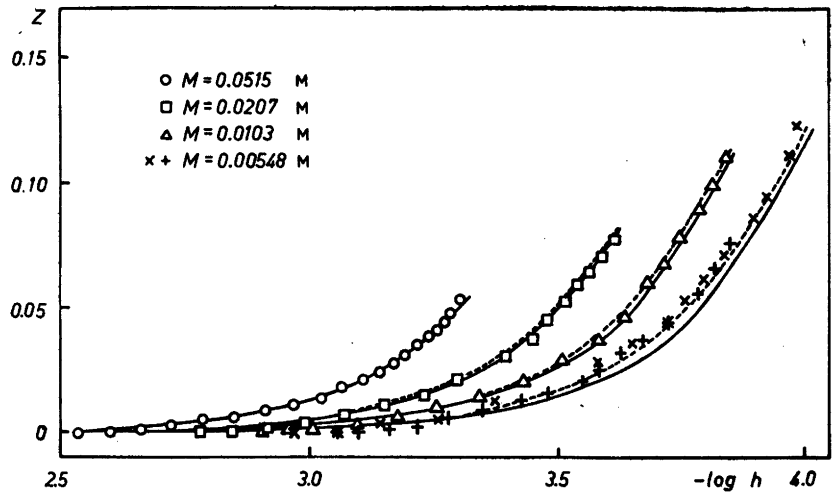

Fig. 2. Average number, Z, of hydroxyl groups bound to each vanadyl ion as a function of log h. O, □, △, +, ×, *are experimental points for different total concentrations,* M, *of vanadium. Full curves calculated for* $\varkappa_{1,1} = 0$, $\varkappa_{2,2} = 10^{-6.88}$. *Dashes calculated for* $\varkappa_{1,1} = 10^{-6.0}$, $\varkappa_{2,2} = 10^{-6.88}$

Table 1. Data Z, *log* h

M = 0.0515 M		M = 0.0207 M		M = 0.0103 M		M = 0.00548 M			
−log h	Z	−log h	Z	−log h	Z	−log h	Z	−log h	Z
2.534	0.000	2.721	0.000	2.904	0.000	3.053	0.000	2.972	0.001
2.598	0.000	2.781	0.000	2.958	0.001	3.101	0.000	3.053	0.0015
2.661	0.001	2.845	0.001	3.027	0.002	3.159	0.001	3.144	0.004
2.721	0.003	2.917	0.002	3.095	0.004	3.218	0.0025	3.259	0.005
2.784	0.005	2.992	0.004	3.174	0.007	3.278	0.006	3.374	0.013
2.850	0.006	3.073	0.007	3.253	0.011	3.348	0.009	3.582	0.028
2.912	0.009	3.152	0.011	3.343	0.015	3.425	0.013	3.653	0.036
2.970	0.011	3.232	0.015	3.430	0.021	3.472	0.016	3.720	0.044
3.024	0.014	3.301	0.021	3.507	0.029	3.544	0.021	3.763	0.053
3.068	0.018	3.396	0.030	3.582	0.037	3.883	0.025	3.798	0.061
3.107	0.021	3.443	0.037	3.636	0.047	3.626	0.032	3.847	0.071
3.139	0.024	3.479	0.045	3.683	0.061	3.671	0.037	3.900	0.085
3.171	0.028	3.516	0.052	3.717	0.068	3.722	0.044	3.927	0.094
3.190	0.031	3.541	0.059	3.751	0.079	3.781	0.056	3.974	0.111
3.217	0.035	3.558	0.063	3.785	0.089	3.815	0.066	3.986	0.123
3.239	0.038	3.589	0.070	3.813	0.100	3.847	0.076		
3.256	0.041	3.612	0.077	3.841	0.111				
3.262	0.044								
3.277	0.047								
3.303	0.053								

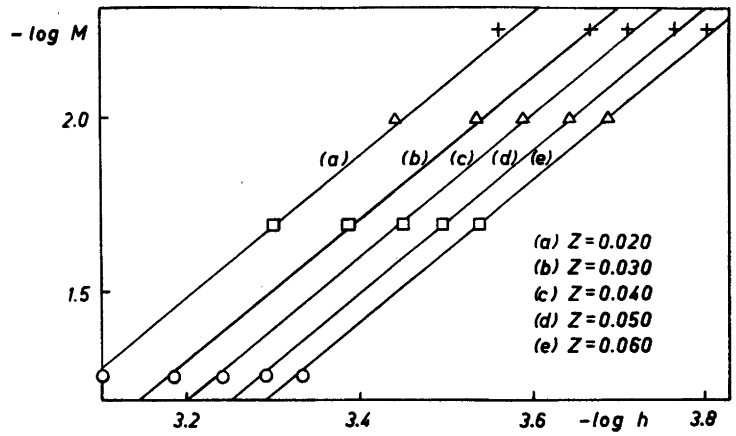

Fig. 3. Log M *as a function of log* h, *for different values of* Z. *The best lines of slope 2.0 are shown.*

and all points would lie on the same Z (log h) curve, regardless of the value of M. As this is not the case, at least one polynuclear hydroxyl complex must be formed.

Now the system may contain any species of the general formula $(VO)_q(OH)_p^{(2q-p)+}$, where p and q may have any positive integral values. A quite general method for computing the hydrolysis constants

$$\varkappa_{p,q} = [(VO)_q(OH)_p^{(2q-p)+}][H^+]^p/[VO^{2+}]^q$$

in systems of this type has been developed by Hedström [25]. Unfortunately, as measurements of the concentration of free VO^{2+} could not be made, measurements at a very large number of concentrations of total metal would be needed. Hedström's approach was therefore not used in the present work, and the data were analysed to see if they could be explained by imposing certain restrictions on the values of p and q. It has previously been found that the hydrolysis products of a number of metal ions, Me, have the general "core+links" formula $Me[(OH)_tMe]_n$, here t and n are integers, such that t is a constant, but that n may vary. The treatment of systems of this type, in which p is a linear function of q, has been developed by Sillén [26,27] (*cf.* parts VIII—XII of this series [11–15]).

If all the complexes present in appreciable amounts can be written in a "core+links" form, a set of parallel Z (log h)$_M$ curves should be obtained. The value of t is then given by

$$t = (\partial \log M/\partial \log h)_Z \qquad (5)$$

It is seen in Fig. 2 that the data for the hydrolysis of vanadium(IV) do lie on parallel Z (log h)$_M$ curves. A value of $t = 2$ was obtained from Fig. 3, in which log M is plotted against log h for several values of Z. Again, if only the species

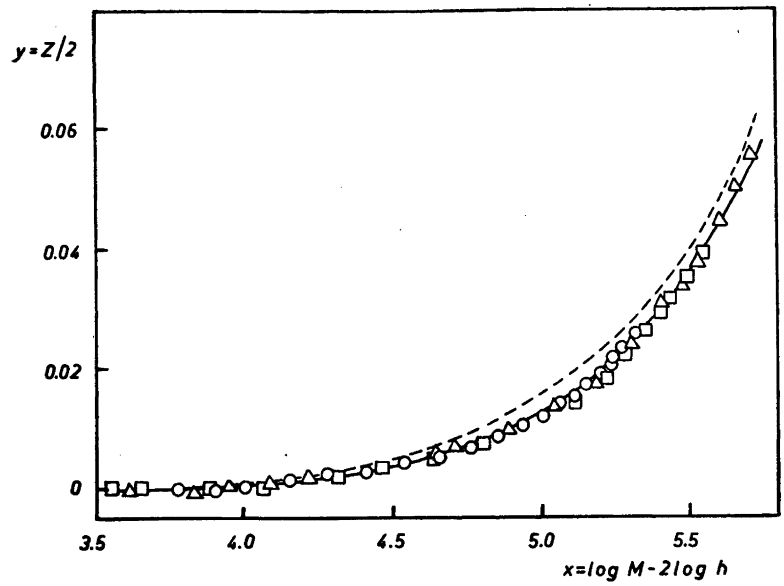

Fig. 4. y *(* = Z/2*) as a function of* x *(* = *log* M — 2 *log* h*).*
The symbols are those used in Fig. 2. Dashes represent the experimental curve for M = *0.005 M. The full curve is calculated for* $\varkappa_{1,1} = 0$, $\varkappa_{2,2} = 10^{-6.88}$

$VO((OH)_tVO)_n$ are formed, all experimental points should lie on the same curve $y(x)$, where

$$y = Z/t \tag{6}$$

and

$$x = \log M - t \log h \tag{7}$$

The experimental curve, $Z/2$, ($\log M - 2\log h$) is shown in Fig. 4, and it is seen that points for 0.05 M $\gg M \gg$ 0.01 M do lie on a single curve. The small deviations which are observed for the points at the lowest concentration of vanadium (broken curve in Fig. 4) may indicate the presence of mononuclear complexes (see p. 1189). However, on account of the limited accuracy of the glass electrode, and of the very low values of Z (< 0.1) obtained, it was not considered practicable to investigate the behaviour of more dilute solutions of vanadium(IV).

Now as y is a function of x only, and as $t = 2$, any complexes formed in appreciable amounts are of the type $VO((OH)_2VO)_n$ or $VO(O.VO)_n$. Direct analysis of the curve $y(x)$ should indicate whether n is a constant or a variable.

Direct analysis of the curve $y(x)$

It has been shown (Ref.[27], equations 14 and 15) that the function $y(x)$ may be used to calculate

$$\log (1 + g) = \int_{-\infty}^{x} y \, dx + \log (1-y) + y \log e \tag{8}$$

and $$\log u = x - y \log e - \int_{-\infty}^{x} y \, dx \quad (9)$$

Here $$u = [VO^{2+}]h^{-2} \quad (10)$$

$$g = \sum k_n u^n \quad (11)$$

and k_n is the equilibrium constant for the reaction

$$(n+1)\ VO^{2+} + 2n\ H_2O \rightleftharpoons VO((OH)_2VO)_n^{2+} + 2n\ H^+$$

The average number of $((OH)_2VO)$ or (O.VO) "links" per complex is given by

$$\bar{n} = \mathrm{d} \log g / \mathrm{d} \log u = \sum n\ c_n / \sum c_n \quad (12)$$

where c_n is the equilibrium concentration of the complex $VO((OH)_2VO)_n^{2+}$.

A smooth curve was drawn through the experimental points y, x for 0.05 M $\gg M \gg$ 0.01 M, and a set of values of $\int_{-\infty}^{x} y \, dx$ was obtained by graphical integration. Values of $\log(1+g)$ and $\log u$ were calculated using equations (8) and (9), and are shown in Table 2.

Table 2. Direct analysis of the curve y(x).

y	x	$10^3 \int_{-\infty}^{x} y\, dx$	$\log u$	$\log (1+g)$
0.01	4.895	3.389	4.887	0.00337
0.015	5.080	5.696	5.068	0.00565
0.02	5.210	7.957	5.194	0.00787
0.025	5.310	10.219	5.289	0.01008
0.03	5.400	12.690	5.375	0.01249
0.035	5.478	15.230	5.448	0.01496
0.04	5.540	17.541	5.505	0.01718
0.045	5.592	19.742	5.553	0.01928
0.05	5.640	22.019	5.596	0.02145
0.055	5.685	24.374	5.633	0.02369

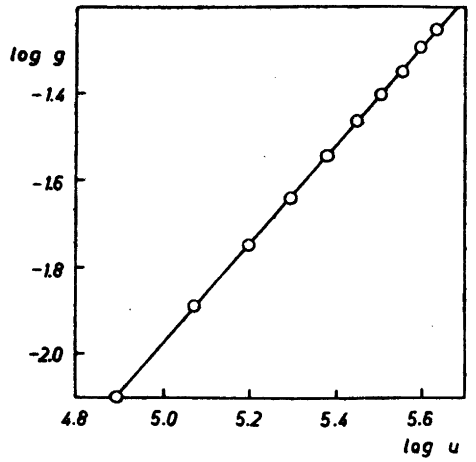

Fig. 5. Direct analysis of the curve y(x); *log* u (= *log* $[VO^{2+}]h^{-2}$) *as a function of log* g (= *log* $k_n u^n$).

The residual integral $\int_{-\infty}^{3.9} y.\,dx$ was assumed to be zero (*cf.* Fig. 4). The function log g (log u) was calculated, and is plotted in Fig. 5. A straight line of slope 1.1 was obtained over the whole range studied ($0 \leqslant y \leqslant 0.05$). A slope of 1.0 would indicate that only the first complex, $VO((OH)_2VO)^{2+}$ is formed. The slightly higher value obtained is not considered to be significant; as Hietanen [13] has emphasized, $\bar{n}$ is very sensitive to small errors in y in the range $y<0.2$. If higher complexes were formed, the value of d log g/d log u would be expected to increase with increasing u. No such curvature was observed.

If only the first complex is formed, then from equation (11)

$$\log g - \log u = \log k_n \qquad (13)$$

and the value of log k_n may be obtained from equation (13) for each of the points log g, log u. The value

$$\log k_1 = \log \varkappa_{2,2} = -6.90 \pm 0.02$$

was obtained using points in the range $0.02<y<0.05$ (see below).

Other methods for calculating the hydrolysis constant

If, as indicated by direct analysis of the curve $y(x)$, only the first "core+links" complex is formed, then p and q are constants, such that $p = t$, and $q = 2$. For a system containing only one complex, $Me_Q(OH)_P$, it may be shown that

$$\log \{P^{Q-1}Z(P-QZ)^{-Q}\} = \log \varkappa_{P,Q} + (Q-1) \log M - P \log h \qquad (14)$$

Whence $(\partial \log M/\partial \log h)_Z = P(Q-1)$ (15) (*cf.* Ref.[26] equation 29).

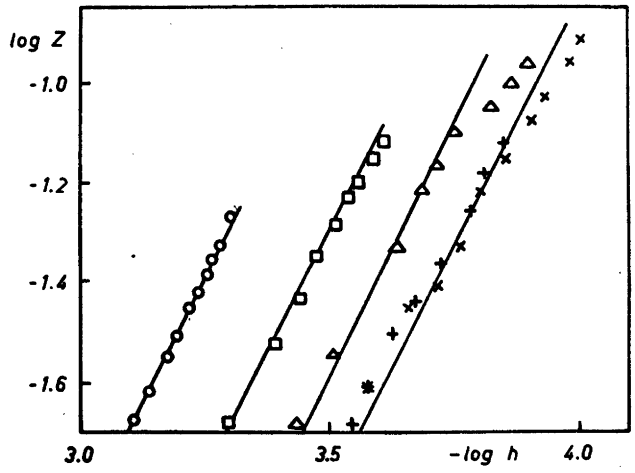

Fig. 6. Log Z as a function of log h, showing the best straight lines of slope 2.0. The symbols for experimental points are those used in Fig. 2.

Combining equations (5) and (15),

$$P / (Q-1) = t$$

Furthermore, $\lim_{Z \to 0} (\partial \log Z / \partial \log h)_M = -P$ (17)

Plots of log Z against log h are shown in Fig. 6. Points for very low values of Z (<0.02) are omitted, as the value of log Z is very sensitive to small experimental errors in Z over this range. Limiting slopes of —2 were obtained for the concentrations of metal 0.05 M$\ll M \ll$0.01 M, indicating that $P = 2$. As $t = 2$ (see p. 1184), equation (16) gives $Q = 2$.

When $P = 2$ and $Q = 2$, equation (14) reduces to

$$\log \{Z / 2 (1-Z)^2\} = \log \varkappa_{2,2} + \log M - 2 \log h \qquad (14a)$$

Thus, if $Me_2(OH)_2$ is the only complex formed, a plot of log $Z/2(1-Z)^2$ against (log M — 2 log h) should be a straight line of unit slope and of intercept log $\varkappa_{2,2}$. The data for the hydrolysis of the vanadyl ion are plotted in fig. 7, and it is seen that points for 0.05 M$\gg M \gg$0.01 M do lie on a straight line of unit slope. This is further strong indication that the only complex formed in appreciable amounts is $(VO)_2(OH)_2^{2+}$ or $(VO)_2O^{2+}$. The intercept gives $\log \varkappa_{2,2} = -6.87 \pm 0.02$. Deviations are again observed for points at the lowest concentration of vanadium.

The experimental curve $y(x)$ may also be compared with theoretical curves, calculated for different values of $\log \varkappa_{2,2}$, using equation (14a). A good fit was

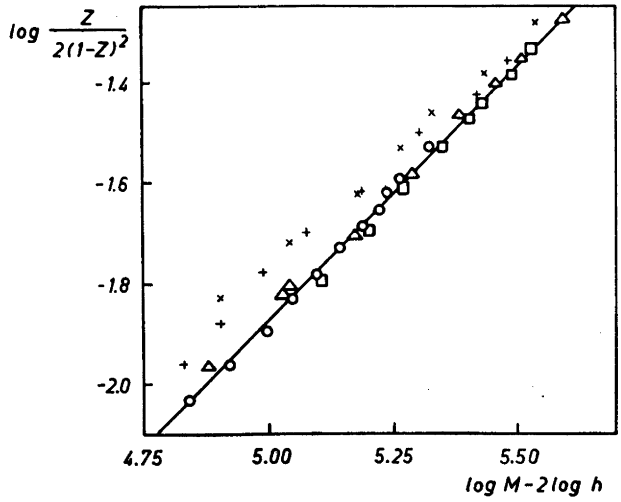

Fig. 7. Log {(Z/2 (1—Z)²} as a function of (log M — 2 log h). The best straight line of unit slope is drawn through the experimental points for 0.05 M$\ll$M$\ll$0.01 M. The symbols are those used in Fig. 2.

obtained with $\log \varkappa_{2,2} = -6.88$ (see Fig. 4). It may be shown that equation (14a) is identical to that used by Sillén ([27], equation 23) for the case where only one complex, $Me((OH)_2Me)$ is formed (hypothesis II, $N = 1$).

The possible presence of hydrolysis products other than $(VO)_2(OH)_2^{2+}$

(a) Higher polymeric species. In order to test whether higher polymers, *e. g.* $VO((OH)_2VO)_2^{2+}$, are present in appreciable amounts, the shape and position of the experimental curve $y(x)$ was compared with curves calculated for the simplified cases suggested by Sillén [27]. A good fit was obtained with some of the curves calculated assuming that n may vary, and that the ratio k_{n+1}/k_n is independent of the value of n ([27], hypothesis IIIa). It was found that $k_0 \geqslant 0.5$, where $k_0 = k_n k^{-n}$, and $k = k_{n+1}k_n^{-1}$. For a given value of k_0, the value of k may be calculated from the experimental data. Combination of equations given previously (Ref.[27], equations 33, 36, 37) leads to

$$k^2\,[y^2\,10^{2x}\,(1-y)^2] - k[2y^2 10^x(1-y) - k_0 10^x(1-2y)^2] + y^2 = 0 \qquad (18)$$

Using equation (18), values of $k_1 = k_0 k$ were calculated for some of the possible values of k_0, and are given below.

k_0	$\log k_1 = \log \varkappa_{2,2}$	mean $\log \varkappa_{2,2}$
0.5	-6.90 ± 0.02	
1.0	-6.88 ± 0.03	-6.88 ± 0.04
2.0	-6.86 ± 0.02	
20.0	-6.90 ± 0.02	

Thus for $k_0 \geqslant 0.5$, the value of k_1 is independent of the value of k_0 within the limits of experimental error. The very good agreement between this value of k_1, and the values obtained by other methods, indicates that no higher hydroxyl polymers are present in appreciable amounts. (*i. e.* $\sum_{n=2}^{n=\infty} c_n M^{-1} \leq 0.3\,\%$) This would be expected at the very low values of Z (<0.1) studied.

(b) Mononuclear species. Figs. 2, 4, 6 and 7 indicate that the experimental data for $M \sim 0.005$ M cannot be explained satisfactorily by assuming that only the complex $(VO)_2(OH)_2^{2+}$ is present. The observed deviations may be explained in terms of the formation of appreciable amounts of the mononuclear complex, $VO.OH^+$, at this concentration of vanadium. If the species $(VO)_2(OH)_2^{2+}$ and $VO.OH^+$ are the only two complexes in solution, it may be shown that

$$h = \left(\frac{1-Z}{2Z}\right)\left\{\varkappa_{1,1} + \sqrt{\varkappa_{1,1}^2 + 8Z\varkappa_{2,2}M}\right\} \qquad (19)$$

Sets of curves $Z(\log h)_M$ were calculated for different values of $\varkappa_{1,1}$ using equation (19), and assuming that $\log \varkappa_{2,2} = -6.88$. The experimental data agree satisfactorily with the curves calculated assuming that $\log \varkappa_{1,1} = -6.0 \pm 0.1$ (*cf.* broken curves in Fig. 2).

DISCUSSION

Potentiometric and absorptiometric studies indicate that vanadium (IV) exists as the vanadyl ion, VO^{2+}, in the range of acidity 2.95 M $\gg h \gg$ 0.002 M. The potentiometric titration data for $h <$ 0.002 M can be explained by assuming that only two further complexes, $VO.OH^+$ and $(VO)_2(OH)_2^{2+}$, are formed. As might be expected for the low values of Z ($<$0.1) at which precipitation occurs, there is no evidence for the formation of higher polymeric species in solution. Values of the equilibrium constant, $\varkappa_{2,2}$, for the reaction

$$2VO^{2+} + 2H_2O \rightleftharpoons (VO)_2(OH)_2^{2+} + 2H^+$$

have been calculated by a number of methods, and are shown below.

Method	$\log\varkappa_{2,2}$
Direct analysis of curve $y(x)$	-6.90 ± 0.02
Equation (14a)	-6.87 ± 0.02
Curve-fitting. Hypothesis II	-6.88 ± 0.02
Hypothesis IIIa, $k_0 \gg 0.5$	-6.88 ± 0.04

The good agreement obtained using different methods of calculation is further evidence that no higher complexes are formed in appreciable amounts.

The equilibrium constant for the reaction

$$VO^{2+} + H_2O \rightleftharpoons VO.OH^+ + H^+$$

was calculated primarily from the data for $M \sim 0.005$ M. The value obtained ($\log\varkappa_{1,1} = -6.0 \pm 0.1$) indicates that, even at this low concentration of metal, only 0.2 % of the total vanadium is present as $VO.OH^+$ when $Z = 0.05$. Unfortunately, the value of $\varkappa_{1,1}$ could not be calculated from data for lower concentrations of vanadium, as Z and h could not be measured sufficiently accurately in the range $M <$ 0.005 M. The constant obtained is of the same order as that calculated by Meites [4] for infinite dilution.

As two hydroxyl groups cannot be distinguished from one —O— group by equilibrium measurements in a constant ionic medium, the vanadyl ion may be either VO^{2+} or $V(OH)_2^{2+}$. Similarly, there are a number of possible structures of the hydrolysis products $[V(OH)_3 + sH_2O]^+$ and $[V_2(OH)_6 + s'H_2O]^{2+}$, where s and s' are positive or negative integers, such that $s \geqslant -1$, and $s' \geqslant -3$. Thus several complexes with different values of s and s' may coexist, and isomers may also be formed. Although it has been found that the vanadyl ion is monomeric in solid vanadyl sulphate [28], no crystallographic data on the structures of basic vanadyl salts are available. It is therefore difficult to assign probable structures to the hydroxyl complexes formed in solution.

The hydrolysis products of several metals may be written as $Me((OH)_2Me)_n$, (*i. e.* as "core + links" complexes, with $t = 2$). When Me represents the iron (III) [10,11], copper (II) [18], or vanadyl ion, only the first complex,

$Me((OH)_2Me$, has been detected. In the case of scandium [15], indium (III) [16], bismuth [11,19] and uranyl [12] ions, a series of complexes is formed. Values of the equilibrium constant, $\varkappa_{2,2}$ for the reaction

$$2\ Me^{2+} + 2H_2O \rightleftharpoons Me((OH)_2Me)^{2+} + 2H^+$$

are listed below for cases where Me is a doubly charged ion.

Ion	$\log\varkappa_{2,2}$
UO^{2+}	− 6.05
Cu^{2+}	−10.6
VO^{2+}	− 6.88

The product of hydrolysis of the vanadyl ion is somewhat less stable than that of the uranyl ion, but is very much more stable than the corresponding copper complex. As would be expected, the hydroxyl complexes derived from doubly charged ions are much less stable than those derived from the trivalent ions of the B sub-group metals indium [16] and bismuth [11,19], and of the transition metal, iron [10,11].

The dimerisation constant for the species $VO.OH^+$ is given by

$[(VO_2(OH)_2^{2+}] / [VO.OH^+]^2 = \varkappa_{2,2} / \varkappa_{1,1}^2 = 10^{5.1\pm0.1}$

Dimerisation constants for the hydroxyl complexes, MeOH, of some other metal ions are given below.

MeOH	$\log (\varkappa_{2,2}/\varkappa^2_{1,1})$	Ref.
$CuOH^+$	+ 5.4	18
$FeOH^{2+}$	+ 3.2	10
$InOH^{2+}$	+ 3.7	16
$ScOH^{2+}$	+ 3.8	15

It is seen that the dimerisation constant of the singly charged vanadium(IV) complex, $VO.OH^+$, is of the same order of magnitude as that of the analogous copper(II) complex, but is considerably higher than the dimerisation constants of the doubly charged species derived from iron(III), indium(III) and scandium.

It is of interest that, for the copper(II), uranyl, and vanadyl complexes, the $Me(OH)_2$ "link" of the polynuclear species is uncharged, and the ratio of metal ions to hydroxyl groups is the same as in the precipitate. An approximate value of the solubility product, S.P., of the vanadium (IV) precipitate was obtained from the values of Z and h at which visible precipitation first occurred. At this point, the value of the term $[VO^{2+}]h^{-2} = M(1-Z)h^{-2}$ was found to be $10^{5.5\pm0.3}$, and the solubility product is given by

$$\text{S.P.} = \text{M}(1-Z)h^{-2}K_w{}^2$$

where K_w is the ionic product of water. Then as $K_w = 10^{-14.2}$ in a 3 M perchlorate medium[29] S.P.$\sim 10^{-23.0}$.

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On the Stereochemical Structure of Cytochrome c

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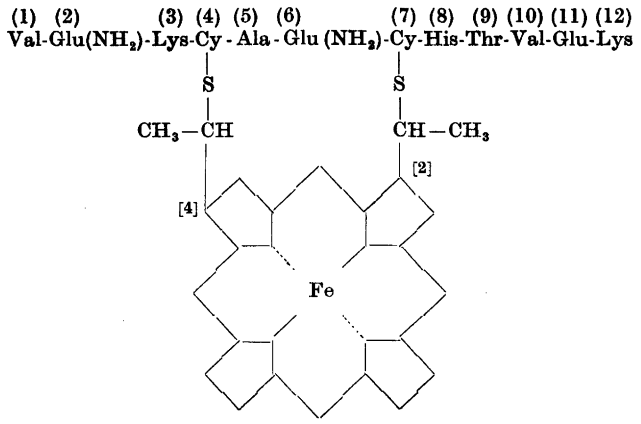
The haemopeptide from cytochrome c of Tuppy and Paléus contains 11 amino acid residues of known sequence, three of them linked to the haem moiety. This substance offered an unusual possibility of investigating the structure of the peptide chain by means of models. It was found that an α-helix structure is very probable. The histidine imidazole then forms a strainless covalent bond with the iron atom, and the axis of the α-helix is parallel with the plane of the haem. Further evidence suggested that the same is true for cytochrome c.

Sedimentation experiments on the haemopeptide showed that it is monomer in acid solution but polymer at alkaline reaction. The polymerisation is due to intermolecular haemochromogen bonds, and is reversed by histidine. The light absorption curve between 500 and 600 mμ of this di-histidine haemochromogen was indistinguishable from that of ferrocytochrome c. A "half" haemochromogen is supposed to exist at slightly acid reaction.

The partial specific volume of cytochrome c was calculated from determinations of its amino acid composition. Comparison with the experimental value indicated a compact structure in which the haem contributes very little to the volume. A "rhombic" packing of peptide chains, distorted to a "square" packing around the haem is suggested.

Earlier work in this institute has established that the haem of cytochrome c is linked to the protein moiety by two thioether bonds between cysteine residues and the α-carbon atoms of the vinyl side chains in the positions [2] and [4] of the haem disc [1–3]. Physico-chemical research made it likely that haemochromogen-forming groups are imidazoles of histidine residues [4,6], and Margoliash [6] has recently presented additional evidence in favour of at least one of the haemochromogen-forming groups being histidine.

The investigations of Tuppy and Bodo [7], and Tuppy and Paléus [8] on different haemopeptides obtained by partial hydrolysis of cytochrome c with proteolytic enzymes have established the following amino acid sequence in beef and salmon cytochrome c:



The sequence (4)—(12) was found to be exactly the same in horse and pig cytochrome c, whereas the chicken cytochrome c contained serine instead of alanine at position (5) — an interesting case of a chemically verified species difference in a protein [8].

The haemopeptide obtained by peptic degradation of beef cytochrome c contains the amino acids (1) to (11). Paléus, Ehrenberg and Tuppy [9] have studied this peptide in its ferric state by means of spectrophotometry, titrimetry, and magnetometry. They found one acid form with essentially ionic bonds to the iron and one alkaline form with covalent bonds to the iron and giving a haemochromogen spectrum upon reduction. The two groups linked to the ferric iron appeared to attach themselves stepwise with pK'-values of 3.4 and 5.8. The intermediate form, with only the first group titrated, had spectral and magnetic properties of intermediate character. At that time nothing could be said with certainty about the character of the bonds with the iron. It was assumed that the group titrated with pK' 3.4 is the imidazole residue of histidine (8) and that the other group with pK' 5.8 is either the α-amino group of valine (1) or the ε-amino group of lysine (3).

Experimental proof of the mode of hydrogen bonding in protein molecules has hitherto practically exclusively been furnished by X-ray crystallography. This method has not yet been applied in the case of cytochrome c *. However, this peptic degradation product of cytochrome c provides a compound of known amino acid sequence that is linked to a rigid structure (the haem-disc) by two chemically well-defined thioether bonds, and in addition, in neutral or alkaline solution, by covalent haemochromogen bonds. This offers a unique possibility of investigating which structures could be compatible with the restrictions introduced by the bonds between the peptide and haematin moieties. The feasibility of the different "spiral" or "pleated sheet" conformations recently suggested as alternatively present in protein molecules [10] could then be compared.

* However, see addendum.

We have therefore studied the possible hydrogen bonded structures of the haemopeptide by means of steric models. The degree of polymerization of the haemopeptide at different pH was studied in the ultracentrifuge. The α- and β-bands of the ferrohaemopeptide were measured spectrophotometrically under various conditions. A complete amino acid analysis of cytochrome c was made, and the results used for calculating the partial specific volume for comparison with the unusually low experimental value which indicates a particularly compact structure. The results were also correlated with previous data on the haemopeptides and cytochrome c. Short summaries have already been published [11].

MATERIAL AND METHODS

About 1.5 mg of a haemopeptide was used in the ultracentrifugal and spectrophotometrical experiments. The sample was one of those used by Paléus, Ehrenberg and Tuppy [9]. It had been obtained by peptic degradation of beef cytochrome c and contained 2.76 % iron.

Beef cytochrome c was kindly put at our disposal by Dr. S. Paléus. It was prepared according to Paléus and Neilands [12] and contained 0.433 % iron.

In the spectrophotometric studies we have also used a sample of protohaemin with 8.26 % iron. The amino acids used were obtained commercially.

All the spectrophotometric experiments were made at 20° C in a Beckman Spectrophotometer, Model DU.

The sedimentation experiments were made in a Spinco Ultracentrifuge, Model E. Because of the low molecular weight of the haemopeptide we used the synthetic boundary cell manufactured by Spinco. All experiments were made at 59 780 r.p.m. which corresponds to about 250 000 g in the middle of the cell where the boundary was formed. Because of the intense colour of the substance (on a weight basis its absorption is more than six times stronger than that of cytochrome c) special precautions had to be taken. We used a red glass filter at the slit, Schott-Jena R G 1, 2 mm, allowing only light of wavelengths longer than 600 mμ. With dilute solutions of the haemopeptide the light absorption was then sufficiently low to give enough blackening of the plate where the light had passed through the peptide solution, without overexposing the other part of the plate. The high diffusion rate of the haemopeptide made it advisable to use the highest concentration possible. The experiments therefore had to be carried out within a narrow concentration range of about 1 mg peptide per ml solution. With a slit width of 0.3 mm, a bar angle of about 30° and exposure intervals of 4 min., a good series of pictures could be obtained with exposure times of 30 *s* in alkaline and 60 *s* in acid solutions. Ten to fifteen exposures were taken in every experiment.

In preliminary experiments on cytochrome c it was found difficult to produce good synthetic boundaries between the solvent and solutions with 1 mg or less protein per ml. As we had only about 1.5 mg of the haemopeptide at our disposal, which was sufficient for only five experiments, it was necessary to insure the formation of good boundaries in each experiment. A sufficient stabilizing effect during the formation period was obtained by adjusting the content of sodium chloride in the peptide solution to 10.5 mg/ml and in the solvent, to be layered above, to 9.5 mg/ml. The layering usually occurred at 6 000 r.p.m. and the first exposure was taken at full speed some ten minutes later. At that time the high diffusion rate of sodium chloride had already flattened out the gradient curve due to the salt, so that it did not influence the position of the superposed and still sharp peptide gradient curve.

As a consequence of the low concentration and sedimentation velocity, and the high diffusion rate and light absorption of the haemopeptide, special precautions had to be taken in evaluating the results. Two different methods were used. In the first procedure the plate was placed in a common photographic magnifying apparatus and projected in the scale 10/1 on a paper where the image was copied with a pencil. On these drawings the position of the peak in comparison with the meniscus and the index could be estimated. Three persons evaluated every plate independently. In the other method a Hilger micro-

photometer for spectrographic plates was used as a comparator. The surface with the entrance slit to the photocell was used as a projection screen on which the images of peak and reference lines were successively brought to coincide with the slit by adjustment of the micrometer on which the differences were read. In both methods the sedimentation rates were calculated from the peak positions according to the method of independent intervals. When the means were calculated the double weight was given to the values obtained by the comparator method, which is considered to be the more objective one, and to those obtained from the drawings of the person who was specially trained in drawing and evaluating the sedimentation diagrams.

Professor L. Pauling generously presented to us a new type of steric bond models for building peptide chains with coplanar peptide bonds, in the scale 1 Å = 1 inch. A haem model was made from a metal disc in the same scale. All C—C and C—N distances were made 1.34″, the pyrrol rings as regular pentagons, and the angle between the bonds to the methine carbon atom 116°. This resulted in a N—Fe distance of 1.9″ in accordance with the known data for covalent radii [13]. The imidazole ring was represented by a regular pentagon with sides 1.42″. Side chain models were manufactured from bent metal rods and suitable joints allowing free rotation around the axis of the bonds.

RESULTS

Sedimentation experiments

The results of the sedimentation experiments are presented in Table 1, and some exposures are reproduced in Fig. 1. From the mean values of S_t the sedimentation constants referred to standard conditions S_{20}°, were obtained after proper correction for viscosity and density as described by Pedersen [14]. In these calculations we have used the mean concentration of sodium chloride, 10 mg/ml, and an estimated value of the partial specific volume of the haemopeptide of 0.7.

The sedimentation constant of beef cytochrome c obtained by the present technique is about 10 % in excess in comparison with earlier data determined with the same centrifuge [15]. This increase might be due to the salt gradient. We have not investigated the question further.

The diffusion coefficients were calculated as follows. From the drawn copies the maximal height, H_{max}, was read, and its inverse square was plotted *versus* the time, which gave an approximately straight line with slope K. The surface A under the curve was measured and an apparent diffusion coefficient

Table 1. Summarized results of the sedimentation experiments on beef cytochrome c and its peptic haemopeptide (Php). The standard errors of S_{20}° are given.

Expt. No.	Sample	Buffer	pH	S_t med $\times 10^{13}$	S_{20}° $\times 10^{13}$	D $\times 10^{7}$	M	f/f_0
1	Php	0.03 M citrate	2.3	0.53	0.57±0.08	26.5	2 000	1.08
2	Php	0.03 M citrate	2.3	0.57	0.62±0.06	21.5		
3	Php	0.05 M borax	8.7	1.54	1.57±0.04	11.8	10 800	1.25
4	Php	0.05 M borax + 0.145 M histidine	8.7	0.76	0.85±0.04	30	[2 300]	[0.83]
5	Php	0.05 M acetate	4.0	2.34	2.53±0.20	56	[3 800]	[0.38]
6	Cyt-c	0.05 M borax	8.7	1.70	1.83±0.06	(11.3)	13 600	1.21

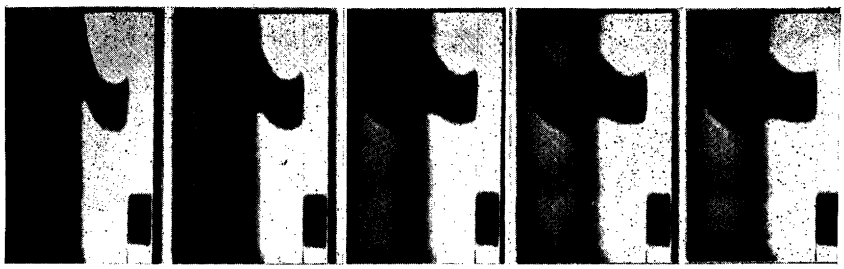


Fig. 1. Sedimentation experiment 2 of Table 1. Exposures taken 4, 12, 20, 28 and 36 min. after full speed had been attained. Exposure time 1 min. Full speed, 59 780 r.p.m. was reached 8 min. after layering.

could be obtained by the formula $D_{app} = \frac{A^2K}{4\pi}$. In case of cytochrome c, D_{app} was about twice as high as the value of 11.3×10^{-7} cm^2. s^{-1} determined by Pedersen [16]. Experiments on cytochrome c without a stabilizing salt gradient revealed that this difference was only partly an effect of the salt gradient. By means of the values of D_{app} the diffusion coefficients of Table 1 have been calculated in proportion to Pedersen's value for cytochrome c.

The molecular weights and the frictional ratios were calculated according to the well known formulae [14]

$$M = \frac{R\ T\ S}{D(1-V\varrho)} \text{ and } \frac{f}{f_0} = \left(\frac{1-V\varrho}{D^2SV}\right)^{1/3} \times 10^{-8}$$

The formula of the haemopeptide gives a molecular weight of 1 880 if one hydroxyl is assumed to be bound to the iron. The iron content of 2.76 % corresponds to the value of 2 020. Our results of experiments 1, 2 and 3 reported in Table 1 show that the haemopeptide is monomer at low pH and polymerized at alkaline reaction. The reasonable values of the frictional ratios and the molecular weight of the monomer indicate that the diffusion coefficients are of the right order and that the species are essentially monodisperse both at pH 2.3 and 8.7. The molecular weight of the polymer is estimated to be 10 800, indicating a pentamer or a hexamer.

In experiment 4 histidine was added to a sample at pH 8.7. The decrease of the sedimentation constant shows that histidine has a depolymerizing effect. The depolymerisation is not complete, which is reflected in a too high diffusion coefficient and a too low value of the frictional ratio.

The sample of experiment 5 was dissolved in dilute acetic acid and then brought to pH 4.0 by addition of a measured amount of sodium hydroxide. The peptide appeared to be polymer and polydisperse at this pH.

Spectrophotometric investigations

Tsou [17] determined the absorption spectrum of his peptic degradation product, which was somewhat less pure than our sample. He found that the spectrum of the ferrous form was "identical with that of ferrocytochrome c in

Table 2. Light absorption data of cytochrome c and some of its degradation products in the reduced state. $\varepsilon = \frac{1}{c} \cdot \frac{1}{d} \log \frac{I_o}{I}$, *where c in mM and d in cm.*

Sample and source	Peptic haemopeptide from beef cytochrome c			Cytochrome c from beef	Cytochrome c from horse	Tryptic haemopeptide from horse cytochrome c		
Author	Tsou[17]	Our data		Our data	Tuppy & Bodo[7]	Tuppy & Bodo [7]		
pH	7.3	8.9	8.9	8.9	7.3	7.3	7.3	7.3
In presence of	—	—	0.14 M histidine	—	—	—	1 000-fold excess of α-benzoyl-L-histidine	Extrapolated to infinite excess of α-benzoyl-L-histidine
ε_{550}	28.0	26.9	27.0	26.9	28.0	15.0	23.4	24
ε_{520}	14.5	13.6	15.8	15.9	16.4	9.4	14.6	15
$\varepsilon_{550}/\varepsilon_{520}$	1.93	1.98	1.71	1.69	1.71	1.60	1.60	1.60

the visible region and in neutral and alkaline solutions". However, a close examination of his absorption curve reveals that this conclusion is not entirely justified: the ratio between the heights of the α- and β-bands is a little higher than in ferrocytochrome c. Our preparation also showed the same property, see Table 2. It appeared to us that the difference might be due to the fact that the haemopeptide in contradistinction to cytochrome c contains only one imidazole residue per iron atom. We therefore investigated the light absorption upon addition of histidine. As seen from Fig. 2 and Table 2 the histidine

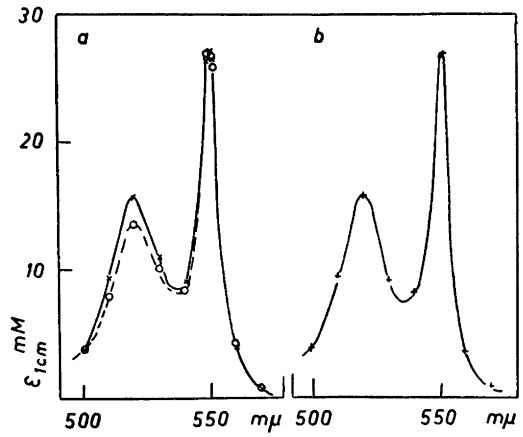


Fig. 2. a) Absorption spectrum of reduced peptic haemopeptide at pH 8.9 (O) and at the same pH with 0.14 M histidine added (×).
b) Absorption spectrum of reduced beef cytochrome c. The concentration of the haemopeptide was determined as pyridine haemochromogen in 0.1 N NaOH + 25 % pyridine + $Na_2S_2O_4$. The same extinction was assumed as for cytochrome c under corresponding conditions.

increased the height of the β-band, giving the same ratio $\varepsilon_\alpha/\varepsilon_\beta$ as cytochrome c itself. Thus the peptic haemopeptide gives α- and β-bands that are indistinguishable from those of cytochrome c only after the addition of histidine.

Tuppy and Bodo [7] examined a tryptic degradation product in the same spectral region at pH 7.3. They found that this haemopeptide alone gave rather low α- and β-bands which increased on the addition of histidine or α-benzoyl-L-histidine. We have extrapolated their data graphically to infinite concentration of α-benzoyl-L-histidine. It is seen from Table 2 that the band heights never reach those of cytochrome c, and that the ratio $\varepsilon_\alpha/\varepsilon_\beta$ remains constant at a somewhat lower level than for cytochrome c.

The peptic haemopeptide contains three potential haemochromogen-forming groups: α—NH_2 in Val(1), ε—NH_2 in Lys(3) and imidazole in His(8). We considered it of interest to compare the ability of such groups to form compounds with ferri- or ferro-protoporphyrin at pH 7, since we have not been able to find such data in the literature. It was found that no spectrophotometrically observable compounds were formed with lysine or glycyl-glycine. This was not unexpected since the pK' of primary amino groups is between 8 and 10.5. Thus at pH 7 they are practically entirely in the $-NH_3^+$ form which cannot combine with haematin iron. Histidine, on the other hand, with its pK' of the imidazole of 6.1, combined both with ferri- and ferro-protoporphyrin. Each iron atom coordinated simultaneously two histidine molecules, in accordance with the findings of Cowgill and Clark [18], who studied the combination of several imidazoles with ferri-mesoporphyrin. The dissociation constants of the two compounds were found to be $K_{ox} = 0.004$ M^2 and $K_{red} = 0.015$ M^2.

Partial specific volume of cytochrome c in relation to its amino acid composition

The partial specific volume of cytochrome c is unusually low, $V = 0.702$—0.707 [16,19]. It was considered to be of interest to determine whether this is due to an unusually high content of heavy amino acids, or whether the haem part is perhaps so intimately connected with the protein part that its contribution to the molecular volume is reduced.

The partial specific volume, V, has been calculated for many proteins from their amino acid composition and the empirical values for the partial specific volumes of the different amino acid residues [20,21]. The values were always in very satisfactory agreement with those obtained by the direct determination of V on the proteins.

In horse haemoglobin the haem part does not seem to have any great influence on V; the value calculated from the amino acid composition, disregarding the haem, was 0.741, the experimentally observed value 0.749 [21].

We now calculated V for horse myoglobin using both Tristram's [22] and our unpublished data on the amino acid composition. V was found to be 0.744 and 0.742, respectively, in close agreement with the experimental value 0.741 [23].

Beef cytochrome c with 0.433 % Fe was analyzed for the amino acid contents according to a modified Stein and Moore procedure. The results are given in Table 3.

Table 3. Amino acid composition of beef cytochrome c with 0.433 % Fe and M = 12 900.

Amino acid residue	Number per molecule	Amino acid residue	Number per molecule
Aspartic acid	9.4	Leucine	6.2
Serine / Threonine	8.5	Tyrosine	3.8
		Lysine	18.3
Glutamic acid	11.9	Histidine	3.1
Glycine	15.5	Arginine	3.4
α-alanine	6.6	Phenylalanine	3.3
Valine	3.3	Methionine	2 *
Proline	3.9	Cysteine	2 *
Isoleucine	6.2	Tryptophane	1 **

* According to Åkeson [24] and Paléus [25].
** According to Theorell and Åkeson [4].

V calculated from these values was = 0.737, thus considerably higher than the experimentally found V = 0.702—0.707. If we assume that the haem part does not increase the molecular volume, but only the weight, V would be = 0.700. The haem thus seems to occupy a space within the protein molecule, where it fits in without expanding the molecule to any large extent.

Structural Models

It was assumed that all the amino acids had the L-configuration. Both left and right hand screws of the α, π, and γ-helices [10] (3.7, 4.4 and 5.1 amino acids per turn, respectively) were constructed. "Pleated sheet" structures were also considered.

We first investigated the possibilities of attaching the haem part to the peptide chain by the two thioether bonds. As seen from the formula (p. 1194) there are two alternatives to be considered: A) Cy(4) to haem side chain [4], Cy(7) to [2], and B) Cy(4) to [2], Cy(7) to [4].

All of the four combinations between γ-helices and the haem were found to be very improbable because of strong steric hindrance. When the peptide chain was formed as an element of a "polar pleated sheet" the thioether bonds could not at all be attached; and when it was formed as required by the "parallel" or "antiparallel" pleated sheet configurations, the attachment of the thioether bonds resulted in distortion of the chain. These structures were therefore considered to be very unlikely.

Thioether bonds could readily be formed with either α- or π-helices.

We now investigated the possibilities of attaching haemochromogen-forming nitrogen atoms to the iron. The free amino group of valine (1) was always too far away from the iron; and it is of small interest, since it is very probably not free in cytochrome c. The histidine imidazole or the ε-amino group in lysine(3) could in many cases be attached to the iron, but never simultaneously from opposite sides of the haem. Because of the flexibility of the lysine side chain its ε-amino-group could easily be attached to the iron in all

Table 4. Comparison of the feasability of the different α- and π-helix structures for the haemopeptide.

Alternative No.	Helix type	Screw type	Cysteine 4 linked to α-carbon	Imidazole imino group pointing towards amino acid	Angle between helix axes and haem plane	Strain for linking imidazole to haem iron
1	α	left	4	1	<5°	none
2	»	»	4	11	30°	some
3	»	»	2	1	15°	none
4	»	»	2	11	45°	some
5	»	right	4	1	<5°	none
6	»	»	2	1	<5°	none
7	π	left	4	—	70°	much
8	»	»	2	—	70°	much
9	»	right	4	—	60°	some
10	»	»	2	—	80°	very much

the α-helix alternatives, but less easily in the π-helices. However, since there are manifold reasons to believe [4–6] that imidazoles are the haemochromogen-forming groups in cytochrome c, and we have already shown in this paper that lysine is unable to form proto-haemochromogen at neutral reaction, we focussed our interest on the imidazole group of our haemopeptide. When the imidazole was attached to the iron by a bond, perpendicular to the plane of the haem, rigid structures were formed. In the case of left-handed α-helices we observed that two slightly different structures were possible, both with the imidazole disc essentially parallel with the axis of the helix, but with the free imino group pointing either towards amino acid (1) or (11).

The results with the α- and π-helices are summarized in Table 4. It is seen that the π-helices are all unfavourable for attaching imidazole to the iron. Not only is the bond formation possible only with considerable strain; but it further-

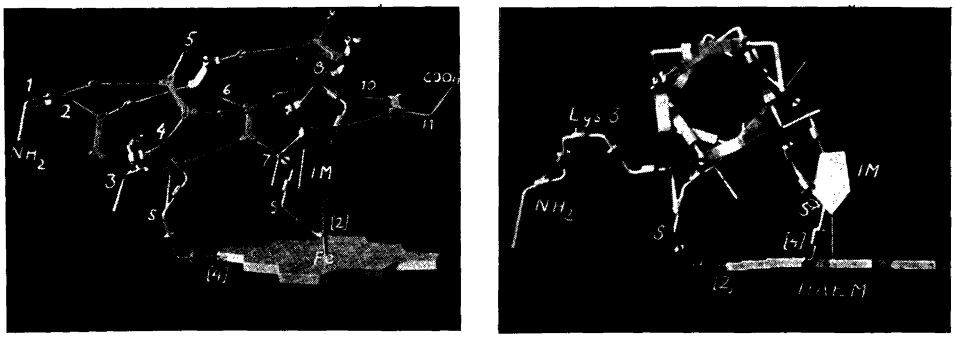


Fig. 3. Pictures showing the structure model of the haemopeptide with left handed α-helix corresponding to alternative 1 of Table 4. a) perpendicular to the helix axes and b) along the helix axes. Figures without brackets indicate the α-carbon atoms of the corresponding amino acid residues. Figures in brackets indicate the substitution positions of the haem.

more results in structures where the haem plane forms angles of 60—80° with the axis of the helix. For spatial reasons such structures seem less likely to occur in cytochrome c than such with the haem parallel to the helix axis. If this argument is applied to the α-helix structures, the most probable alternatives are numbers 1, 5 and 6 (Table 4). And finally, if the left-hand screw is preferred because it has been found to occur in a great number of proteins [26,27], whereas right hand helices do not seem yet to have been found, we are left with alternative 1 as the most likely one. Fig. 3 shows this structure from the side and along the helix axis.

DISCUSSION

When haemochromogens are formed in solution under usual conditions the attachment of one group to the iron on one side of the haem disc so greatly favours the coupling of another on the opposite side that the reaction appears to be trimolecular. Therefore, "half" haemochromogens have never been observed.

The haemopeptide from cytochrome c offers an interesting example of a molecule where the intramolecular formation of a half haemochromogen should be strongly favoured. It contains one histidine residue in an optimal position for coordination to the iron; after this bond has been formed no nitrogenous groups are left that can easily be used to complete the haemochromogen in slightly acidic solution. The spectrophotometrical transition with a pK' of 3.4 and $n = 1$ [9] is thus likely to be a reflection of the imidazole forming a half haemochromogen. A water molecule or an anion is likely to be bound to the iron on the opposite side. The paramagnetic susceptibility $\chi_m = 8\,800 \times 10^{-6}$ c.g.s. e.m.u.[9], indicates dsp^2 bonds with 3 odd electrons to be present.

A second, spectrophotometrically operable transition occurs at higher pH ($pK = 5.8$, $n = 1$), resulting in a complete covalent structure with 1 odd electron [9]. Upon reduction haemochromogen bands appear which are very similar, but not identical with those in ferrocytochrome c (See Fig. 2). Primary amino groups are here coordinated opposite to the imidazole, because no other groups likely to form covalent bonds with the iron are present. The pK' of the free amino group in a valylpeptide is likely to be about 8. A shift of this value to 5.8 in the haemopeptide is plausible as a result of the much higher stability of the covalent d^2sp^3 structure than that of the "half" haemochromogen structure. The ε-amino group of lysine with its pK' about 10.5 is less likely to take part in this reaction. A d^2sp^3 structure cannot be formed with the aid of the imidazole and an amino group belonging to the same molecule, unless the peptide chain is unfolded. If the peptide chain is an α-helix, as proposed, the iron atom must therefore be bound to the imidazole of the same molecule and a primary amino group of another; in neutral or alkaline solution polymerization would be expected. This was found to be the case; for some reason a monodisperse penta- or hexamer is formed. The aggregates were depolymerized upon addition of histidine. This indicates that the free histidine competitively replaces the amino group, thus forming a di-histidine haemochromogen with α- and β-bands that are spectrophotometrically indistinguishable from those of cytochrome c.

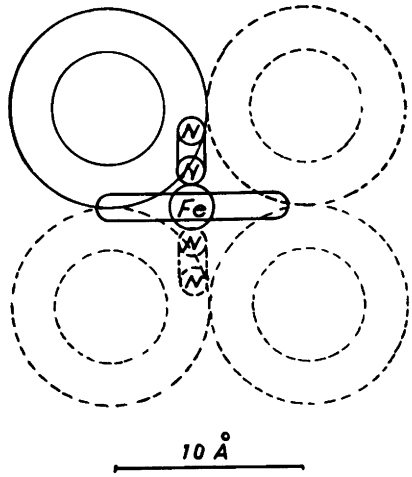


Fig. 4. Schematic axial view of proposed structure with four parallel α-helices in "square" packing around the haem. Full-drawn portion corresponds to haemopeptide. Space between inner and outer circles is occupied by side chains.

The peptic haemopeptide precipitates from solutions of low ionic strength between pH 4.3 and 7.4 [9]. It contains seven groups that are titratable from pH 2 to pH 11. Four of these are carboxyl groups, one imidazole and two primary amino groups. Furthermore, for calculating the isoelectric point the positive charge of the iron atom has to be considered. The haemopeptide should therefore be isoelectric when four equivalents are titrated starting from pH 2. According to Paléus *et al.*[9] this occurs at pH 5.4. Their data indicate that precipitation takes place when the molecule bears less than 1 ½ plus or minus charges. We believe that this precipitation is essentially isoelectric in character and not necessarily connected with the arrangement of the haemochromogen-forming groups.

There are good reasons to believe that the steric structure of the peptide fragment studied here really forms a part of native cytochrome c. The α-helix is in itself the most probable of the peptide helices, because it gives the most compact structure with minimal van der Waals energy [10]. The parallelity of the helix axis and the haem disc makes it possible to arrange peptide chains in the close packing that is necessary in order to explain the low partial specific volume of cytochrome c. If the side chains of the α-helix structure presented in Fig. 3 are considered, a cylinder with an average diameter of 10.5 Å results [10]. This occupies just one quadrant of a plane perpendicular to the plane of the haem disc. It is plausible to imagine that other helices in cytochrome are arranged parallel to each other as indicated in Fig. 4. Two haem-linked imidazoles are shown, one belonging to the haemopeptide part, the other to another part of the protein.

The proposed structure certainly avoids any steric hindrance, but the "square" packing of the helices is not the closest possible arrangement, and would result in a partial specific volume as high as 0.73 if it is assumed to extend over the whole length of the molecule. In an α-helix the axial projection per amino acid residue is about 1.5 Å [10]. Since there are about 100 resi-

dues in cytochrome c, the length of the molecule would be 35—38 Å, if four parallel helices of equal length are assumed. If the helices are packed in the closest possible manner, "rhomboic" packing, and the "square" packing is only a local distortion effected by the presence of the haem, which has an axial projection of 10—12 Å, then the theoretical partial specific volume comes out as 0.71 in satisfactory agreement with the experimental data.

The structure shown in Fig. 4 would shield the haem iron from direct contact with, for example, oxygen, carbon monoxide or cyanide, in accordance with the known properties of cytochrome c. On the other hand, the electron exchange with suitable donors and acceptors must in some way be facilitated. On both sides of the haem the non iron-bound imino nitrogen of the haem-linked imidazole has a rather exposed position in the crevice between two α-helices. An active group of another protein, *e. g.* the iron of DPNH cytochrome c reductase [28] could perhaps coordinate such a nitrogen atom. If the iron is present as a non-haem chelate compound this coordination would be sterically possible. The electron transfer could then be imagined to take place through the resonating bonds of the imidazole ring. A similar reaction mechanism was previously suggested for the reduction of cytochrome c by means of leucoflavins or hydrogen [29].

The oxidation of ferrocytochrome c does not seem to be possible by direct contact between cytochrome oxidase haem iron and the haem-linked imidazole in cytochrome c, because of steric hindrance. But there is a possibility that an oxygen molecule, activated by being linked to cytochrome oxidase iron, could come close enough to be able to accept electrons from cytochrome imidazole.

The proposed structure and the reaction mechanisms discussed of course remain hypothetical and need further experimental confirmation. It is especially desirable to obtain crystalline cytochrome c in order to carry out detailed X-ray investigations. The type of helix, however, could perhaps be determined on amorphous material according to the X-ray technique used by Riley and Arndt [26,27].

Addendum May 6, 1955: Our results have been summarized in a forthcoming chapter of "*Currents in Biochemical Research*" (manuscript sent 17 February, 1955) and in a letter to the *Nature* of 30 March, 1955. When the present manuscript was ready a paper by Arndt and Riley (*Phil. Trans.* 247 A, p. 409, 24 March, 1955) came to our knowledge. These authors have classified cytochrome c as a closely packed "α"-protein with left hand α-helices, as revealed by their X-ray scattering method. The orientation of the haem *versus* the peptide chains was not discussed. On the basis of our independent results we can now say that our proposed structure must be essentially correct.

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X-Ray Studies on the System BiF_3-Bi_2O_3

I. Preliminary Phase Analysis and a Note on the Structure of BiF_3

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The fluorine content of bismuth fluoride gradually decreases on heating it in air, the end product being bismuth oxide. A preliminary phase analysis of the system BiF_3—Bi_2O_3 has been made. The structure of bismuth fluoride as found by Zachariasen from studies of powder photographs has been confirmed by single crystal methods.

On heating bismuth fluoride in air it is found that the fluorine content gradually decreases at 600—800° C. The reaction is probably $BiF_3 + xH_2O = BiO_xF_{3-2x} + 2xHF$ or, for low values of x, possibly $BiF_3 + xH_2O = Bi(OH)_xF_{3-x} + xHF$. Samples of about 2 g bismuth fluoride were heated in small platinum crucibles for various periods of time (up to 60 h) in an electrical oven at 670° C. This temperature was chosen because it gave a suitable reaction velocity. The fluorine content of the samples was determined and powder photographs were taken in focusing cameras using Cr - K or Cu - $K\alpha$ radiation. The result of the phase analyses is summarized in Table 1. The actual heating times are not related to the progress of the reaction since the humidity of the atmosphere was not controlled.

METHODS OF SYNTHESIS

To prepare BiF_3, we first tried the method of precipitating the compound from a bismuth nitrate-mannitose solution with a potassium or sodium fluoride solution as described by Hassel and Nilssen [4] and by Hund and Fricke [5]. However, the products obtained in this way always contained potassium or sodium and attempts to remove the alkali metal lead to preparations considerably poorer in fluorine (18 %) than the theoretical value calculated for BiF_3 (21.4 %). We therefore tried the simple method of digesting bismuth oxide with dilute (1:1) hydrofluoric acid in a platinum dish. This method lead to samples with 21.1 ± 0.2 % F and 77.4 ± 0.2 % Bi (theoretical 21.4 % F and 78.6 % Bi).

METHODS OF ANALYSES

Fluorine. The samples were distilled with perchloric acid as described by Willard and Winter [6]. The distillate was titrated with thorium nitrate solution, using sodium-alizarin sulphonate as an indicator.

Table 1. Results of phase analyses of various samples obtained by heating bismuth fluoride at 670° C in air.

Heating time (h)	Fluorine content obs., % by weight	Phases (main constituents)
0	21.1	BiF_3*
	20.0	$BiF_3 + \alpha$
	19.7	α
	17.3	$\alpha + \beta$
	14.8	β
	12.5	β
	6.7	BiOF** $+ \gamma$
	5.2	γ
	4.5	δ
	2.7	δ
60	0	Bi_2O_3 ***

* Orthorhombic BiF_3, Ref.[1]
** BiOCl type, Ref.[3]
*** Monoclinic Bi_2O_3, Ref.[3]

α. Approximate composition is $BiO_{0.1}F_{2.8}$. Hexagonal structure of tysonite type with the cell dimensions $a = 4.08$ Å and $c = 7.32$ Å. A report of a determination of the crystal structure will appear shortly.

β Face-centered cubic cell with $a = 5.84$ Å (sample with 14 % F). The composition is probably variable.

γ Seemingly a deformed face-centered cub c lattice.

δ Monoclinic needles with $a = 19.7$ Å, $b = 7.5$ Å, $c = 21.3$ Å and $\beta = 107°$.

Bismuth. The samples were heated with conc. nitric acid and repeatedly evaporated to dryness in a platinum dish to expel the hydrofluoric acid. The residue was then dissolved in dilute nitric acid and the bismuth precipitated with ammonia and finally weighed as Bi_2O_3.

SINGLE CRYSTAL INVESTIGATION OF BISMUTH FLUORIDE

Since the powder photographs of bismuth fluoride, prepared in the way described above, differed completely from those of the cubic compounds described by Hassel and Nilssen [4] and Hund and Fricke [5] and because the paper by Zachariasen (*l. c.* in Ref.[1]) was not known to us at that time we started an investigation of single crystals of our compound by means of rotation and Weissenberg photographs (0*kl*, 1*kl*, *h*0*l*, and *h*1*l*). We found the Laue symmetry to be *Pmmm* and the unit cell dimensions $a = 6.56$ Å, $b = 7.04$ Å and $c = 4.83$ Å, which is in good agreement with Zachariasen's values $a = 6.56$ Å, $b = 7.03$ Å and $c = 4.86$ Å. The observed density was 7.92 and the calculated value for 4 formula units per unit cell 7.90. The systematically absent spectra were those characteristic of the space-groups *Pnma* (No. 62) and $Pna2_1$ (No. 33). The positions of the bismuth atoms were found to be in agreement with the former symmetry, occupying the position 4(*c*): $\pm(x, \frac{1}{4}, z)$, $\pm(\frac{1}{2}-x, \frac{3}{4}, \frac{1}{2}+z)$ with $x = 0.35_3$ and $z = 0.038$. Our observations thus agree with the statements of Zachariasen [1] and of Zalkin and Templeton [1] that BiF_3 is iso-

morphous with YF_3, the coordinates of the yttrium atoms according to the latter being $x = 0.367$ and $z = 0.058$. Reasonable Bi—F distances are also obtained when assuming the same F parameters for BiF_3 as those given for YF_3.

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X-Ray Studies on the System BiF_3-Bi_2O_3

II. A Bismuth Oxide Fluoride of Defective Tysonite Type

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A bismuth oxide fluoride of approximate composition $BiO_{0.1}F_{2.8}$ has been prepared. From single crystal investigations the crystal structure was found to be closely related to that of tysonite.

By heating orthorhombic bismuth fluoride in air at 670° C, various bismuth oxide fluoride phases have been obtained as reported in a previous communication [1]. The first phase observed in this series was also prepared by carrying out the heat treatment at 850° C for a period of a few minutes. When similarly heating bismuth fluoride in a closed crucible no change of the substance occurred. The new phase evidently was not a new modification of bismuth fluoride, as was also confirmed by chemical analysis.

The fluorine content of various samples containing the new phase was found to lie between 19 and 21 %, while the theoretical value for bismuth fluoride is *21.4* %. Assuming the new phase to be a bismuth oxide fluoride this will correspond to the approximate composition $BiO_{0.1}F_{2.8}$.

The substance was colourless and could be obtained as crystal plates of up to about 1 mm length and 0.3 mm thickness. X-ray photographs were taken in a Guinier focusing powder camera using Cu—$K\alpha$ radiation (*cf.* Table 1) and in a Weissenberg camera with Cu—K radiation.

UNIT CELL AND SPAGE CROUP

In the single crystal photographs, the reflexions $0kl$, $1kl$, hhl, and $h,h+1,l$ were registered. The Laue symmetry proved to be $6/mmm$. Systematically missing spectra were hhl with l = odd, which is characteristic of the space-groups $P6_3/mmc$ (No. 194), $P6_3mc$ (No. 186) and $P\bar{6}2c$ (No. 190). Powder photographs of a sample prepared at 850° C (20.3 % F) gave the unit cell dimensions $a = 4.08_3$ Å and $c = 7.32_3$ Å.

Preparations of lower fluorine content seemed to show minor alterations in the positions of the reflexions. Some of the lines in the photographs of these

Table 1. Powder photograph of BiO_xF_{3-2x}. Cu-Ka radiation. Sample with 20.3 % F prepared at 840° C. The value of x is 0.09.

I obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl*	I obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl*
m	442	443	002	m	2 895	2 888	203
m	468	475	100	m	3 197	3 199	114
vst	580	586	101	m	3 249	3 245	105
m	914	918	102	vw	3 320	3 327	210
broad m	1 418	1 426	110	m	3 435	3 438	211
m	1 471	1 472	103	w	3 671	3 674	204
vw	1 776	1 773	004	w	3 773	3 770	212
m	1 869	1 869	112	vw	3 999	3 999	006
vw	1 893	1 901	200	w	4 278	4 278	300
m	2 002	2 012	201	m	4 328	4 324	213
w	2 251	2 248	104	vw	4 464	4 474	106
w	2 340	2 344	202	m	4 677	4 671	205
				m	4 714	4 721	302

samples were obviously split up into doublets while others were broadened. This effect and the result of the chemical analyses seem to indicate a distortion of the structure and a variable composition of the phase. For a sample prepared at 840° C with 19.6 % F, a was found to be 4.09_8 Å and c 7.27_7 Å for the pseudo-cell. So far the nature of the slight distortion has not been elucidated and thus, when discussing the structure, the non-deformed atomic arrangement has been considered.

Cell content

For the cell content the three following possibilities must be taken into consideration:

1. The composition of the phase may be represented by the expression $Bi(O, F)_3 \cdot xBi$, the extra bismuth atoms being inserted in interstices in a $Bi(O, F)_3$ array.
2. The formula of the compound is $Bi(OH, F)_3$ corresponding to a possible uptake of water during the heat treatment (*cf.* Ref.[1]).
3. The composition of the phase is BiO_xF_{3-2x}.

Table 2. Cell dimensions and observed and calculated densities.

Temp. °C	F % by weight	Density obs.	Density 1	Density 2	Density 3	a	c
840	21.1	$8.3_1 \pm 0.06$	8.43	8.36	8.34	4.08_0	7.32_3
840	20.3	8.1_9	8.55	8.34	8.29	4.08_3	7.32_3
840	20.1 *	8.2_6	8.63	8.38	8.32	4.07_2	7.32_9
670	19.6 *	8.2_1	8.70	8.37	8.29	4.07_7	7.31_9
840	19.6					4.09_8	7.27_7

* On calculating the cell dimensions the weak extra line in the vicinity of 110 was neglected.

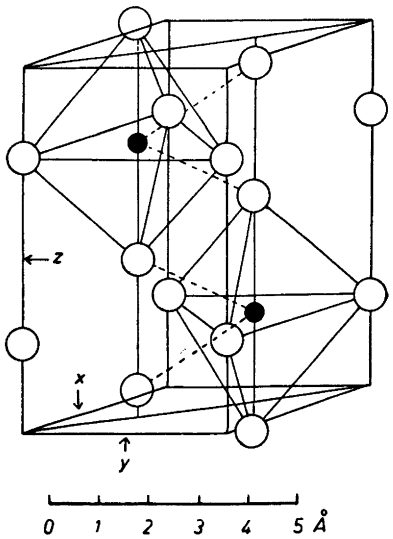

Fig. 1. Unit cell of the BiO_xF_{3-2x} phase, showing the coordination of the Bi atoms (full circles). The dashed lines connect the Bi atoms with (O, F) atoms (open circles, interatomic distances 2.7 Å), the latter belonging to a trigonal prismatic arrangement of non-metal atoms encompassing the $Bi(OF)_5$ trigonal bipyramids, indicated by full lines.

The observed densities for samples of various compositions are given in Table 2 together with those calculated for the alternatives 1—3 assuming the elementary cell or pseudo-cell to contain two formula units. While 1 obviously is in disagreement with the observed data the density measurements do not allow a decision to be made between 2 and 3. However, the high temperature of formation makes it rather improbable that the structure contains hydrogen, while several examples are previously known of bismuth-oxygen compounds possessing vacancies in the oxygen framework. This suggests alternative 3 to be the more probable one.

Crystal structure

(Notation according to International Tables for X-Ray Crystallography, I, Birmingham 1952.)

Since the reflexions $03l$ with l = odd are absent in the photographs, the only possible positions for the two Bi atoms of the unit cell are 2(*d*) and 2(*c*) in space-group $P6_3/mmc$, 2(*b*) in $P6_3mc$ or 2(*c*) and 2(*d*) in $P\bar{6}2c$, the point position of the two latter space groups, however, actually possessing the symmetry $P6_3/mmc$. Intensities calculated for this arrangement of the bismuth atoms are in fair agreement with the observed data. The positions of the light atoms were determined from space considerations, the distance (O, F)—(O, F) being initially assumed to be $\geq$2.50 Å and the distances Bi—(O, F) $\geq$2.20 Å. The former condition is only consistent with the space group $P6_3/mmc$ and the point positions 2(*a*), 2(*b*), 2(*c*), 2(*d*), and 4(*f*) if the symmetry of the light atoms is assumed to be that revealed in the X-ray photographs. The only combination of 6(O, F) atoms on these point positions which is in agreement with the assumed restrictions of the interatomic distances is 2(*b*) and 4(*f*), if the position

2(*c*) is arbitrarily adopted for the bismuth atoms. The *z*-parameter of the latter (O, F) position was finally chosen so as to give Bi—(O, F) distances ≥ 2.3 Å and (O, F)—(O, F) distances ≥ 2.6 Å. In this way the value $z = 0.57$ was arrived at.

Thus the following structure is proposed:

Space-group $P6_3/mmc$ (No. 194)

4 $(O, F)_2$ in 4(*f*): $\frac{1}{3},\frac{2}{3},z$; $\frac{2}{3},\frac{1}{3},\bar{z}$; $\frac{2}{3},\frac{1}{3},\frac{1}{2}+z$; $\frac{1}{3},\frac{2}{3},\frac{1}{2}-z$ $\quad z = 0.57$

2 $(O, F)_1$ in 2(*b*): $0,0,\frac{1}{4}$; $0,0,\frac{3}{4}$

2 Bi in 2(*c*): $\frac{1}{3},\frac{2}{3},\frac{1}{4}$; $\frac{2}{3},\frac{1}{3},\frac{3}{4}$

The corresponding interatomic distances and coordinations will be:

Bi—3 $(O, F)_1$; $(O, F)_1$—3 Bi	2.36 Å
Bi—2 $(O, F)_2$; $(O, F)_2$—Bi	2.35 Å
Bi—6 $(O, F)_2$; $(O, F)_2$—3 Bi	2.70 Å
$(O, F)_1$—6$(O, F)_2$; $(O, F)_2$—3$(O, F)_1$	2.70 Å
$(O, F)_1$—6$(O, F)_2$; $(O, F)_2$—3$(O, F)_1$	3.32 Å
$(O, F)_2$—$(O, F)_2$	2.63 Å
$(O, F)_2$—3$(O, F)_2$	2.70 Å

DISCUSSION

The atomic arrangement thus arrived at is obviously closely related to that of several trifluorides of lanthanides and actinides described by Zachariasen [2] and of the mineral tysonite RF_3 (R = Ce, La *etc.*) studied by Schlyter [3]. It must, however, be emphasized that the present bismuth compound very probably does not exist at the composition BiF_3 but is stabilized by the occurrence of vacancies in the non-metal framework accompanying the substitution of oxygen atoms for a small fraction of the fluorine atoms.

The investigation is continued by studying the cubic bismuth oxide fluoride phase [1] ($a = 5.84$ Å) which is likely to be structurally related to the cubic bismuth fluoride phase described by Hassel and Nilssen [4].

These studies form part of a research program on metal oxides and related compounds financially supported by the *Swedish Natural Science Research Council.*

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X-Ray Studies on Bismuth Oxide Acetate $CH_3COO \cdot OBi$ and Related Compounds

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Bismuth oxide acetate and a series of compounds of approximate composition R·COO·OBi have been prepared. The general arrangement of the atoms seems to be the same as that for BiOCl, the organic anions replacing the chloride ions. A tentative structure is given for the oxide acetate.

The bismuth oxide salts of various fatty acids were prepared using a procedure similar to that given by Hofmann [1] for the synthesis of bismuth oxide acetate. Bismuth oxide was dissolved in the proper acid, the solution diluted with water and the excess acid distilled off. In this way it was possible to synthesize pure specimens of the bismuth oxide salt of acetic acid. Corresponding syntheses of the formate, propionate, *n*-butyrate and *n*-valerate were also performed and gave samples with somewhat high contents of bismuth when compared with the ideal formula of R · COO · OBi. The propionate samples were found to belong to a phase of an extended homogeneity range (*vide infra*). The *n*-butyrate and *n*-valerate were not investigated from this point of view (*cf.* Table 1). The formate and the acetate were obtained as very thin crystal plates. The former was generally discoloured by reduced bismuth while the latter was colourless. The other salts were white powders.

X-ray powder photographs of the various preparations were taken in a Guinier focusing camera using Cu-$K\alpha$ radiation. Parts of the powder photographs of the formate, acetate, propionate, and *n*-butyrate are given in Table 2.

Table 1. Analysis for Bi_2O_3. Observed and calculated densities.

Compound	% Bi_2O_3 obs.	% Bi_2O_3 calc.	Density obs.	Density calc.
BiO · OOCH	88.8	86.3	6.0	5.82
BiO · $OOCCH_3$	81.6	82.0	4.7	4.72
BiO · $OOCC_2H_5$	80.6	78.2	4.1	4.40
BiO · $OOCC_3H_7$	77.8	74.7	3.7	3.93
BiO · $OOCC_4H_9$	73.6	71.4		3.56

Table 2. Part of the powder photographs of bismuth oxide formate, acetate, propionate and n-butyrate.

Bismuth oxide formate

I obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl*	*I* obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl*
m	58	58	001	vw	683	—	—
m	451	451	101	vvw	750	—	—
w	522	520	003	m	787	785	110
vw	555	—	—	w	845	843	111
st	629	624	102	w	914	911	103

Bismuth oxide acetate. Pseudo-tetragonal description.

m	36	37	001	vw	922	924	005
vw	148	148	002	vw	951	—	—
st	416	415	101	vw	969	969	104
vst	523	526	102	vw—	1 091	1 088	113
vw	601	591	004	—	—	—	—
st	709	711	103	w	3 014	3 020	220
st	753	755	110	w	3 034		
st	790	792	111	m	3 773	3 775	310
vw	904	903	112	m	3 788		

Bismuth oxide propionate (80.6 % Bi_2O_3)

vst	26	27	001	st	784	787	110
m	106	107	002	m	812	814	111
w	419	421	101	m	828	821	104
vw	425	427	004	w	896	894	112
st	502	501	102	w	967	962	006
st	637	635	103	w	1 058	1 062	105
w	670	668	005				

Bismuth oxide n-butyrate

st	20	20	001	m	637	—	—
st	78	78	002	vw	668	—	—
w	175	176	003	m	703	706	006
vw	312	314	004	vw	721	—	—
vw	410	—	—	st	788	792	110
m	443	—	—	vw	815	—	—
vw	447	—	—	vw	835	—	—
w	477	—	—	vw	896	—	—
w	487	490	005	vw	954	960	007
m	520	—	—	vw	987	—	—
vw	540	—	—	vw	1 048	—	—
vw	580	—	—				

The powder photographs of the salts of formic, acetic and propionic acids could be completely interpreted by assuming tetragonal or pseudo-tetragonal unit cells with $a \sim 3.9$ Å and c-axes increasing from the formate to the propionate (*cf.* Table 3). In the powder photograph of the acetate most of the lines *hkl* (h and $k \neq 0$) were found to be doubled. No corresponding splitting up of the lines could be detected in the powder photographs of the formate and propionate. The cell dimensions of the propionate were found to vary with its

Table 3. Cell dimensions, bismuth parameters and distances within the $Bi_2O_2^{2+}$ layers.

Compound	a Å	c Å	V Å^3	z_{Bi}	Height of the Bi atom above $z = 0$
BiO · OOCH	3.89	10.16	154	0.12_4 [1]	1.26 Å
BiO · $OOCCH_3$	3.97	12.68	200	0.10_0 [1]	1.27
BiO · $OOCC_2H_5$	3.89	14.92	225	0.08_3 [2]	1.24
BiO · $OOCC_3H_7$ *	3.9	17.4	264	0.07_0 [3]	1.22
BiO · $OOCC_4H_9$ *	3.9	20.0	304	0.06_3 [3]	1.26

[1]) Calculated from Weissenberg photographs.
[2]) Calculated from powder photographs of a sample of 80.6 % bismuth content.
[3]) Calculated from powder photographs, 00l row.
* Dimensions given correspond to 00l and $hk0$ reflexions.

content of bismuth. Thus, for a sample with 81.8 % Bi_2O_3 the dimensions were $a = 3.88$ Å and $c = 14.78$ Å and for a sample with 80.6 % Bi_2O_3 $a = 3.89$ Å and $c = 14.92$ Å.

The powder pattern of the *n*-butyrate was considerably more complicated than those of the lower fatty acid salts. Some of the lines appeared to be grouped in multiplets (*cf.* Table 2). However, the majority of the lines of major intensity could also here be accounted for as $hk0$ or 00l reflexions from a basic unit cell of tetragonal symmetry with $a = 3.9$ Å and $c = 17.4$ Å. Attempts to perform a complete interpretation of the diagram have been without success. The real unit cell may be a distorted modification of the tetragonal unit or it may be a multiple of it. Some of the extra lines may of course be due to impurities.

The *n*-valerate pattern was less complicated than that of the *n*-butyrate and could only be observed up to *d*-values of about 1.36 Å. All except some weak lines could be interpreted as $hk0$ and 00l reflexions from a tetragonal unit cell of dimensions $a = 3.9$ Å and $c = 20.0$ Å.

The conformity of the tetragonal basic unit cells suggests a structural analogy between the various compounds. The density determinations (*cf.* Table 1) are in agreement with a cell content of two formula units of R·COO·OBi. A similar structural unit has previously been observed for bismuth oxide chloride BiOCl[2] of PbFCl-type, the structure of which may be described as follows:

Space-group $P4/nmm$ (No. 129)

2 O	at 2(a):	0,0,0; $\frac{1}{2},\frac{1}{2},0$	
2 Bi	at 2(c):	$0,\frac{1}{2},z$; $\frac{1}{2},0,\bar{z}$	$z = 0.171$
2 Cl	at 2(c):	$0,\frac{1}{2},z$; $\frac{1}{2},0,\bar{z}$	$z = 0.650$

It seemed worth-while to investigate whether a similar atomic arrangement was present in the present compounds.

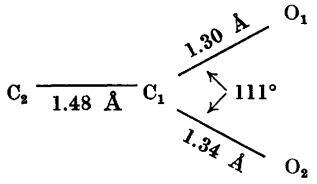
Weissenberg photographs ($h0l$ and $h1l$) were taken of a crystal of the bismuth oxide formate using Cu-$K\alpha$ radiation. The Laue symmetry was found to

be 4/*mmm*. The only systematically absent spectra were *hk*0 with $h+k$ = odd, which is consistent with the space-group $P4/nmm$ (No. 129). Weissenberg photographs of the acetate showed the same symmetry. The only position for the two bismuth atoms in this space-group, consistent with the observed intensities, is in 2(*c*). The values for the corresponding *z* parameters of the various compounds are given in Table 3. The Bi—O distances, assuming 2 oxygen atoms to be situated at 2(*a*), agree well with those found for the BiO layers in other bismuth oxide compounds [3].

The dominating contribution of the bismuth atoms to the intensities of the reflexions makes the determination of the positions of the light atoms an extremely difficult task. Consequently no attempts were made to solve this problem. However, from a knowledge of the cell dimensions and the positions of the bismuth atoms, the following might be inferred:

From space considerations, it is possible to find positions of the organic anions analogous to the chlorine atom positions in bismuth oxide chloride, the anions thus forming double layers interleaving the layers of $Bi_2O_2^{2+}$. Because of electrostatic reasons, it can be assumed that the carboxyl groups are directed towards the $Bi_2O_2^{2+}$ layers. However, several alternatives present themselves and so far it is not possible to decide which is the correct one.

As mentioned above the reflexions *hkl* (*h* and $k \neq 0$) were found to be doubled in the powder photograph of the oxide acetate. The two lines of the doublets were always of the same intensity. This effect could be accounted for by assuming a monoclinic unit cell with $a = c = 3.967$ Å, $b = 12.68$ Å and $\beta = 90.°16$ and the symmetry $P\,2/n$ (no. 13). The corresponding axes for the pseudo-tetragonal cell are $a = 3.967$ Å and $c = 12.68$ Å. A possible atomic arrangement of bismuth oxide acetate consistent with this symmetry is illustrated in Fig. 1. The model for the acetate ion applied here was obtained from the study of zinc acetate dihydrate by v. Niekerk *et al.*[4], *viz.*



The same distances and angles were used here except for the distances $(O_1,O_2)—C_1$ which were assumed to be 1.34 Å. With the monoclinic unit cell and symmetry derived above the Bi atoms would occupy the position 2(*e*): $\frac{1}{4},y,\frac{1}{4}$; $\frac{3}{4},\bar{y},\frac{3}{4}$ with $y = 0.10$ and the two O atoms of the Bi_2O_2-layers would be situated in 2 (*f*): $\frac{3}{4},y,\frac{1}{4}$; $\frac{1}{4},\bar{y},\frac{3}{4}$ with $y = 0$. The two C_1 and C_2 atoms are supposed to lie on the lines $\frac{1}{4},y,\frac{1}{4}$;$\frac{3}{4},\bar{y},\frac{3}{4}$ (*i. e.* at the point positions 2(*e*)) and the $4(O_1,O_2)$ atoms to occupy the position 4(*g*): $\pm(xyz)$; $\pm(\frac{1}{2}-x,y,\frac{1}{2}-z)$. These latter atoms might either be ordered with for instance $x = z = 0.05$ as pictured in Fig.1 or they might be randomly distributed over a series of positions 4(*g*) with $(x—\frac{1}{4})^2 + (z—\frac{1}{4})^2 = (1.11/a)^2$ (1.11 being half the distance $O_1—O_2$ within an acetate ion and $a = c$). In the latter case the problem of the orientation of the

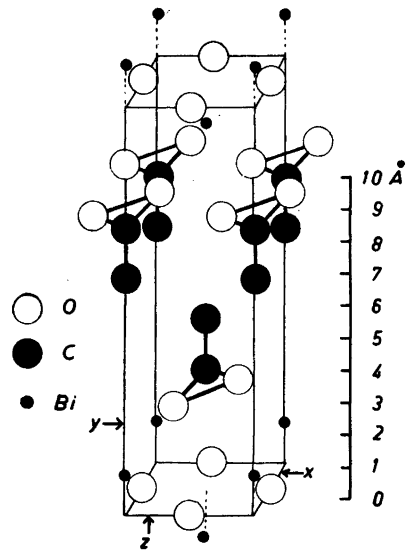


Fig. 1. Tentative structure of bismuth oxide acetate. The origin used in the figure corresponds to the point $\frac{1}{4},0,\frac{1}{4}$ *in the monoclinic cell described in the text.*

carboxyl groups is similar to the problem of the arrangement of the carbonate ions of the minerals bismuthite ($Bi_2O_2CO_3$) and beyerite ($CaBi_2O_2(CO_3)_2$), treated by Lagercrantz and Sillén [5]. The space available requires that the mutual orientation of neighbouring carboxyl groups is ordered while the long range arrangement is a random one. The structures obtained in this way correspond throughout to reasonable interatomic distances, *viz.* $Bi—O_{carboxyl}$ = 2.4 Å for the ordered alternative and 2.4—2.7 Å for the random one while C—C (of the same layer) = 3.97 Å and C—C (of adjacent layers) = 3.6 Å.

It must in this connection be emphasized that the observed symmetry and cell dimensions may be only those of the arrangement of the heavy bismuth atoms, while the actual symmetry of the structures may be lower and the true unit cell larger.

The area available for one $RCOO^-$ group is small, being only about 15 Å^2 as compared with the value 18.2—18.9 Å^2 obtained for potassium soaps (angle of tilt 53—55°) by Lomer [6], the value 17.4 Å^2 obtained by Vand, Aitken and Campbell [7] for silver caproate at 20° C (angle of tilt 73°), both determined by X-ray methods, and the value 21 Å^2 obtained from surface measurements by Langmuir [8] and several other investigators. All of the latter data refer to fairly long chain molecules and it is interesting to note that attempts to prepare bismuth oxide salts of fatty acids higher than *n*-valeric acid and also of *iso*-acids have been without success, and that the syntheses of the *n*-butyrate and *n*-valerate were found to be rather troublesome. It is obvious that only the short chain acids are able to accomodate themselves easily to the restricted mesh of the $Bi_2O_2^{2+}$ layers. A possibility which has not so far been tested is that hydroxyl groups are substituted for a minor proportion of the fatty acid anions of, *e. g.*, the propionate. This arrangement would give a more probable area per fatty acid ion and account for the fact that the unit cells of these

compounds are different from those of the lower fatty acid salts. This hypothetical substitution mechanism might also explain the variable composition and unit cell dimensions observed for the propionate.

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The author wishes to thank Dr. Arne Magnéli for his encouraging interest and for many valuable discussions.

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X-Ray Studies on "Sodium Metabismuthate"

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So-called sodium metabismuthate was prepared using the method of Scholder and Stobbe. X-ray powder photographs indicate that its crystal structure is of the ilmenite type. The Bi parameter was determined. The quadratic form for the lines in the powder photographs is given for a commercial sodium metabismuthate.

So-called sodium metabismuthate ($NaBiO_3$.aq) was prepared using the procedure given by Scholder and Stobbe [1]. The samples were dried over H_2SO_4 *in vacuo*. Water contents between 1.6 and 4.0 % by weight were found, corresponding to a ratio of 0.3—0.6 for H_2O/total bismuth in the samples. The ratio Bi^{5+}/Bi_{total} varied between 88 and 96 %. Analysis of one sample (I) gave: $Na^+:Bi^{5+}:Bi_{total}:H_2O = 1.05:0.96:1.00:0.52$. Powder photographs taken in a Guinier focusing camera using Cu-$K\,a$ radiation indicated a rhombohedral unit cell with $a = 6.21$ Å, $\alpha = 53.°2$ and $V = 143$ Å^3. A few, very weak, extra lines showed that the preparation contained minor amounts of impurities, which, however, could not be identified. No shift in the cell dimensions was found for samples of different compositions. The observed density of (I) was 5.8 in fair agreement with the calculated values of 6.38 for 2 $NaBiO_3$ or 6.58 for 2($NaBiO_3$.0.5 H_2O) per unit cell. The cell dimensions and intensities of the lines in the powder photographs are very similar to those of α-$NaSbO_3$, which was found by Schrewelius [2] to be of the ilmenite type with the unit cell dimensions $a = 6.12$ Å, $\alpha = 51.°1$ and $V = 130$ Å^3. The two compounds are evidently isomorphous. For α-$NaSbO_3$, Schrewelius gave the following data:

Space-group: $R\,\bar{3}$ (No. 148)
Atomic positions: 2 Na at 2(*c*): $\pm(x,x,x)$ with $x = 0.342$
2 Sb at 2(*c*): $\pm(x,x,x)$ with $x = 0.156$
6 O at 6(*f*): $\pm(x,y,z;\ z,x,y;\ y,z,x)$

with $x = 0.54$; $y = -0.03$, and $z = 0.26$.

For the present compound it was only possible to determine the Bi parameter which was found to be 0.160 ± 0.005. In view of the facts that sodium metabismuthate has only slightly larger unit cell dimensions than the water-free α-$NaSbO_3$ and that the positions of the heavy atoms of the two com-

Table 1. Part of the powder photographs of $NaBiO_3$. Cu-Ka radiation.

I obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl* rh	*I* obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl* rh
st	210	210	111	vst	977	978	210
vw	227	—	—	w	1 046	1 047	$11\bar{1}$
st	279	279	100	w	1 115	1 117	200
st	348	349	110	m	1 399	1 398	220
st	631	630	211			1 400	322
vw	677	—	—	vw	1 456	—	—
vw	703	—	—	vw	1 491	—	—
vst	767	768	$1\bar{1}0$	st	1 609	1 608	311
vw	781	—	—			1 609	321
		840	221	m	1 811	1 815	$2\bar{1}0$
m	842	841	222			1 885	$21\bar{1}$
				w	1 888	1 893	333

pounds are very nearly the same, the lattice cannot possibly incorporate the water found in the preparation, which must consequently be combined with the impurities observed. Part of the powder photograph of $NaBiO_3$ is given in Table 1. The investigation evidently shows that $NaBiO_3$ is adequately described as a sodium bismuth(V)oxide.

Commercial "$NaBiO_3.2H_2O$" was also studied but found to be different from the above phase. The yellow preparation "$NaBiO_3.2H_2O$" manufactured by Merck gave rather simple powder photographs which could be explained by assuming a hexagonal unit cell with $a = 5.59$ Å, $c = 7.40$ Å, and $V = 201$ Å^3. Samples with brownish or brownish-black colours, manufactured by Schering-Kahlbaum yielded the same pattern and in addition a number of extra lines. No systematic extinctions were found in the powder photographs. It is worth noting that the hexagonal cell has about the same a axis as $NaBiO_3$ (ilmenite type) when the latter is referred to a hexagonal unit cell with $a = 5.56$ Å and $c = 15.9_5$ Å, which might indicate a structural relationship between the two phases. The Merck sample gave the following composition when analysed: $Na^+:Bi^{5+}:Bi_{total}:H_2O = 0.8:0.8:1.0:5$. The water was calculated by difference. The observed density was 5.0. There are probably two formula units per unit cell of the hexagonal phase, the calculated density being, *e. g.*, 5.2 for $2(NaBiO_3.2H_2O)$ and 6.1 for $2(NaBiO_3.5H_2O)$. The present composi-

Table 2. Part of the powder photograph of "$NaBiO_3 . 2H_2O$" manufactured by Schering-Kahlbaum. Cu-Ka radiation. The extra lines have been omitted.

I obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl*	*I* obs	$10^4\sin^2\Theta$ obs	$10^4\sin^2\Theta$ calc	*hkl*
m	107	109	001	vw	1 009	1 013	200
w	253	254	100	vw	1 121	1 122	201
m	362	362	101	m	1 193	1 192	112
w	432	432	002	vw	1 225	1 229	103
m	686	685	102	vw	1 445	1 451	202
m	758	760	110	m	1 744	1 736	113
m	867	869	111			1 736	104
vw	983	976	003	vw	1 883	1 881	211

tion of this compound can, however, only be found by carrying out a complete structure determination. Part of the powder photograph of "$NaBiO_3.2H_2O$" (Schering-Kahlbaum) is given in Table 2.

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Potentiometric and Spectrophotometric Studies on 8-Quinolinol and Its Derivatives. XIII. Solubility and Ultraviolet Spectra of Some Metal Chelates of 7-Nitro-8-Quinolinol-5-Sulphonic Acid

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The ultraviolet spectra of the three species of 7-nitro-8-quinolinol-5-sulphonic acid and some of its metal chelates have been measured. The solubility products of the chelates of this reagent with eleven divalent metals have been determined and the effect of ionic strength on the solubility investigated. The results are compared with the corresponding results on 8-quinolinol and on some of its derivatives as studied previously by us.

In a previous paper by the present authors [1] the ionization of 7-nitro-8-quinolinol-5-sulphonic acid and the stability of its metal chelates in aqueous solutions were treated. In the present paper the ultraviolet spectra of 7-nitro-8-quinolinol-5-sulphonic acid and those of its metal chelates are dealt with. Like 8-quinolinol-5-sulphonic acid, its 7-nitro derivative has two types of sparingly soluble metal chelates: $M(HA)_2$ and MA. In the first case only the solubility products of $Ba(HA)_2$, $Sr(HA)_2$ and $Ca(HA)_2$ could be determined. In the second case the solubility products of eleven metal chelates were determined.

RESULTS AND DISCUSSION

In Fig. 1 the spectra of the three species of the reagent are represented. In the spectrum of H_2A the peak at 365 mμ (ε = 4 200) obviously corresponds to the peak at 353 mμ (ε = 2 700) in the spectrum of the corresponding species of 8-quinolinol-5-sulphonic acid [2]. This peak occurs in the case of 7-iodo-8-quinolinol-5-sulphonic acid [2] at 353 mμ (ε = 3 420), in the case of 8-quinolinol [3] at 356 mμ and in the case of 5,7-dichloro-8-quinolinol [4] at 368 mμ. The double maximum at 305—325 mμ which is seen in the spectra of many other derivatives of 8-quinolinol and which can be attributed to the pyridine nucleus,

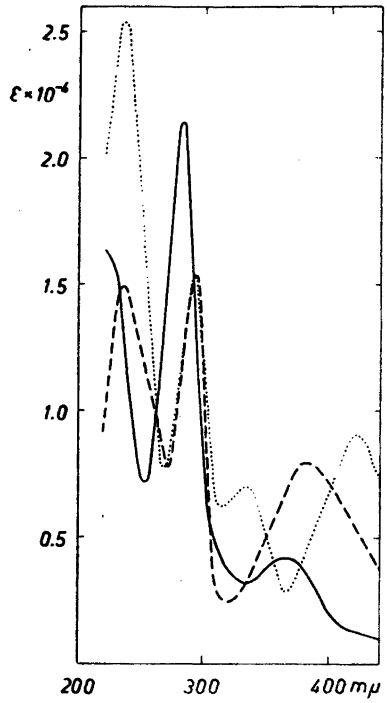

Fig. 1. The ultraviolet absorption spectra of 7-nitro-8-quinolinol-5-sulphonic acid. H_2A, full line, 2 M HCl. HA^-, broken line, pH = 4. $A^=$, dotted line, 0.1 M NaOH.

is not distinctly visible in the present case. The maximum at 284 mμ (ε = 22 800) may correspond to the peak of the spectrum of 5,7-dichloro-8-quinolinol [4] at 260 mμ (ε about 39 000). The other derivatives have peaks at about the same wavelength. The shift to longer wavelengths is thus in the latter case considerable while the peak at 365 mμ has been displaced less.

The spectrum of the species HA^- has three peaks: 238 mμ (ε = 14 900), 293 mμ (ε = 15 300) and 381 mμ (ε = 7 950). The first peak obviously corresponds, for instance, to that of 2-methyl-8-quinolinol [5] at 243 mμ (ε = 35 300) and to that of 5,7-dichloro-8-quinolinol [4] at 247 mμ. The second peak at 294 mμ probably corresponds to that of 5,7-dichloro-8-quinolinol at 277 mμ ($\varepsilon \approx$ 8 300) and to that of 7-iodo-8-quinolinol-5-sulphonic acid [2] at 284 mμ (ε = 20 800). The third peak has obviously no counterparts in the spectra of the other derivatives and this may be attributed to the nitro group. On the other hand the peak at about 300 mμ in the case of many other derivatives has no counterpart in the present spectrum. It may be mentioned that 7-iodo-8-quinolinol-5-sulphonic acid also lacks this peak.

The spectrum of the species $A^=$ has four maxima. The first at 235 mμ (ε = 25 600) obviously corresponds, for instance, with that of 2-methyl-8-quinolinol [5] at 256 mμ (ε = 33 000) and with that of 7-iodo-8-quinolinol-5-sulphonic acid[2] at 260 mμ (ε = 38 000). The second peak at 294 mμ (ε = 15 200) seems to have no counterpart in the spectra of the other 8-quinolinol derivatives. The third maximum at 334 mμ corresponds to the peak at

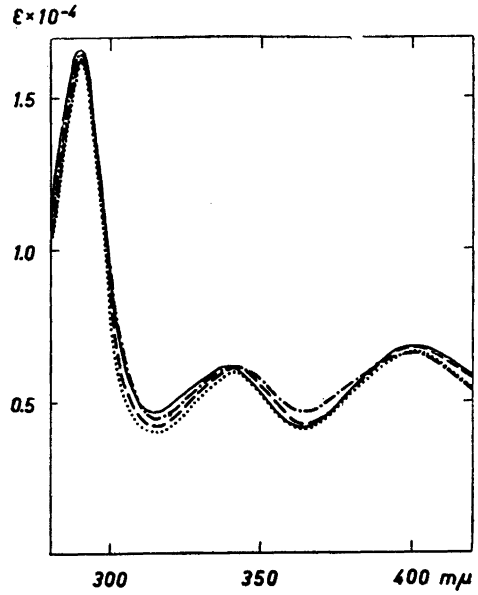

Fig. 2. The ultraviolet absorption spectra of some metal chelates of 7-nitro-8-quinolinol-5-sulphonic acid. Full line: Mn. Broken line: Cd. Dotted line: Zn. Dot- and -dash line: Pb.

335 mμ of the spectrum of 8-quinolinol [3] or to that at 355 mμ in the same spectrum. The fourth peak at 420 mμ (ε = 9 000) has no counterpart in the spectra of the other derivatives.

The spectra of the reagent studied here thus show many peaks which may obviously be attributed to the peaks of the spectra of 8-quinolinol and its other derivatives. However some new peaks also occur.

In Figs. 2 and 3 the spectra of some metal chelates are presented. All the spectra are very similar showing peaks at 290—291 mμ, 340—345 mμ and 395—403 mμ. These spectra are thus rather similar to the spectrum of the ligand $A^{=}$, which has maxima at 293, 333, and 420 mμ. On the basis of the above, it seems obvious that the bond between metal and ligand is chiefly ionic.

The potentiometric method used here for the determination of the solubility products has been previously described [2,6]. As in the case of 8-quinolinol-5-sulphonic acid [6], difficulties arose because of a great tendency to supersaturation. On the other hand the supersaturation makes possible the determination of the stability constants of alkaline earth metal chelates which are very sparingly soluble. A spectrophotometric method was also used. These measurements were made at so high a pH that, according to the values of the stability constants, practically all the reagent was in the form MA. Then the concentration of MA was determined spectrophotometrically using molar extinction coefficients of MA as determined above.

The results on the solubility products

$$[M^{++}]\ [HA^{-}]^2 = S_1 \qquad (1)$$

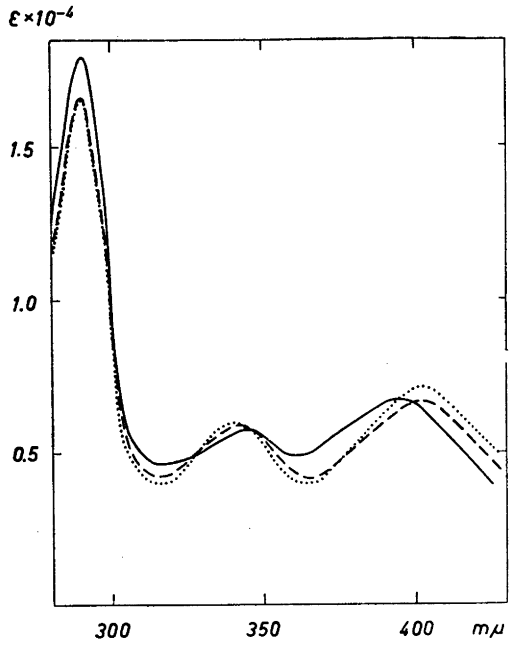

Fig. 3. The ultraviolet absorption spectra of some metal chelates of 7-nitro-8-quinolinol-5-sulphonic acid. Full line: Cu. Broken line: Co. Dotted line: Ni.

are presented in Table 1. Here c_B, c, c_M are the total concentrations of sodium hydroxide, reagent and metal ions, respectively. In Tables 1 and 2 the results concerning the solubility products

$$[M^{++}]\,[A^{=}] = S_2 \qquad (2)$$

are given. The results obtained by the spectrophotometric method are presented in Table 3.

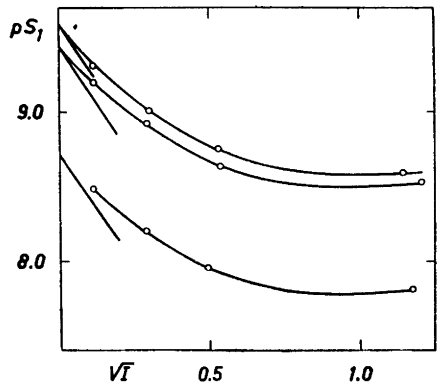

Fig. 4. Solubility products $[Ba^{++}][HA^-]^2$ (lower curve), $[Sr^{++}][HA^-]^2$ (middle curve) and $[Ca^{++}][HA^-]^2$ (upper curve) as a function of ionic strength in solutions of potassium chloride.

Table 1. Potentiometric determination of the solubility products of the barium, strontium and calcium chelates of 7-nitro-8-quinolinol-5-sulphonic acid at 25° C. $S_1 = [M^{++}][HA^-]^2$. $S_2 = [M^{++}][A^=]$.

$Ba(HA)_2$ KCl	pH	$\sqrt{I}$	pS_1	BaA KCl	pH	$\sqrt{I}$	pS_2
$c = 2.18 \cdot 10^{-3}$	2.735	0.103	8.49	$c = 2.02 \cdot 10^{-3}$	3.687	0.155	7.21
$c_B = 0.00$	2.760	0.288	8.21	$c_B = 3.02 \cdot 10^{-3}$	3.803	0.292	6.95
$c_M = 3.22 \cdot 10^{-3}$	2.769	0.487	7.96	$c_M = 7.46 \cdot 10^{-3}$	4.015	0.497	6.58
	2.729	1.170	7.82		4.167	0.732	6.34
					4.241	1.030	6.22
$Sr(HA)_2$	2.718	0.103	9.20	SrA	3.581	0.155	7.36
KCl	2.755	0.279	8.93	KCl	3.736	0.296	7.04
$c = 2.18 \cdot 10^{-3}$	2.780	0.532	8.64	$c = 2.02 \cdot 10^{-3}$	3.842	0.495	6.79
$c_B = 0.00$	2.700	1.197	8.54	$c_B = 3.02 \cdot 10^{-3}$	3.952	0.666	6.60
$c_M = 3.22 \cdot 10^{-3}$				$c_M = 7.45 \cdot 10^{-3}$	3.986	0.881	6.52
$Ca(HA)_2$	2.718	0.101	9.30	CaA	3.276	0.150	8.97
KCl	2.757	0.294	9.03	KCl	3.335	0.300	8.72
$c = 2.18 \cdot 10^{-3}$	2.780	0.520	8.75	$c = 2.02 \cdot 10^{-3}$	3.438	0.505	8.45
$c_B = 0.00$	2.710	1.142	8.61	$c_B = 3.02 \cdot 10^{-3}$	3.479	0.704	8.30
$c_M = 3.05 \cdot 10^{-3}$				$c_M = 6.94 \cdot 10^{-3}$	3.462	0.930	8.27

In the spectrophotometric method described above, a high pH value of the equilibrium solution is necessary. The determination of solubility products at lower pH's by spectrophotometry is also possible. We have used this method in case of the lead chelate. The method was as follows. To the solution of the reagent an about 1.1-fold amount of lead nitrate was added. When the equilibrium was reached, the pH of the solution was determined and the total concentration of reagent in the solution was determined spectrophotometrically as PbA. It was possible to bring all the reagent to PbA by adding sodium hydroxide and lead nitrate in such quantities and as such dilute solutions that precipitation did not occur. The concentration of PbA in the equilibrium solu-

Table 2. Potentiometric determination of the solubility products of the 7-nitro-8-quinolinol-5-sulphonic acid chelates of some divalent metals at 25° C. $S_2 = [M^{++}][A^=]$.

MgA KCl	pH	$\sqrt{I}$	pS_2	MnA KCl	pH	$\sqrt{I}$	pS_2
$c = 13.0 \cdot 10^{-3}$	3.462	0.374	5.56	$c = 2.02 \cdot 10^{-3}$	3.395	0.157	7.67
$c_B = 20.2 \cdot 10^{-3}$	3.868	0.797	5.00	$c_B = 3.02 \cdot 10^{-3}$	3.631	0.430	7.09
$c_M = 46.5 \cdot 10^{-3}$	4.063	1.110	4.76	$c_M = 7.71 \cdot 10^{-3}$	3.716	0.760	6.87
	4.307	1.530	4.61				
CdA				ZnA			
$NaNO_3$	3.141	0.151	8.45	$NaNO_3$	2.710	0.233	8.40
$c = 2.02 \cdot 10^{-3}$	3.242	0.374	7.96	$c = 5.00 \cdot 10^{-3}$	2.761	0.507	8.00
$c_B = 3.02 \cdot 10^{-3}$	3.293	0.770	7.70	$c_B = 7.50 \cdot 10^{-3}$	2.765	0.873	7.82
$c_M = 7.15 \cdot 10^{-3}$				$c_M = 17.1 \cdot 10^{-3}$			
CoA							
$NaNO_3$	2.388	0.356	8.16				
$c = 10.74 \cdot 10^{-3}$	2.650	0.680	7.84				
$c_B = 16.11 \cdot 10^{-3}$	2.274	1.360	8.14				
$c_M = 39.75 \cdot 10^{-3}$							

Table 3. Spectrophotometric determination of the solubility products of some metal chelates of 7-nitro-8-quinolinol-5-sulphonic acid at 25° C. $S_2 = [M^{++}]\,[A^{=}]$.

	$c \cdot 10^3$	$c_M \cdot 10^3$	$[MA] \cdot 10^3$	$\sqrt{I}$	pS_2
MnA	2.077	30.80	0.604	0.304	7.24
KCl	2.077	30.80	0.960	0.610	6.81
CdA	2.077	29.50	0.234	0.295	8.09
$NaNO_3$	2.077	29.50	0.244	0.525	7.80
ZnA	2.079	2.840	0.947	0.410	8.15
$NaNO_3$	9.000	12.29	1.020	0.607	7.89
	9.000	12.29	1.370	0.842	7.85
CoA	1.985	73.5	1.621	0.542	7.86
$NaNO_3$	7.507	277.7	2.490	0.910	7.73
	7.507	277.7	2.940	1.055	7.84
NiA	3.079	2.998	2.691	0.400	8.75
$NaNO_3$	9.000	12.98	3.862	0.625	8.45
	9.000	12.98	4.803	0.833	8.42
CuA	2.077	29.23	1.050	0.296	11.07
$NaNO_3$	2.077	29.23	1.059	0.428	10.89
	2.077	29.23	1.600	0.782	10.66
PbA	2.077	30.40	0.061	0.302	9.36
$NaNO_3$					

Table 4. Spectrophotometric determination of the solubility product of lead chelate in acidic medium. 25° C. Added electrolyte $NaNO_3$.

$c \cdot 10^3$	$c_M \cdot 10^3$	pH	$[A^{=}]$	$[Pb^{++}]$	$\sqrt{I}$	pS_2
5.432	5.975	2.252	$1.13 \cdot 10^{-7}$	$0.81 \cdot 10^{-3}$	0.160	10.03
5.432	5.975	2.304	$5.20 \cdot 10^{-7}$	$1.21 \cdot 10^{-3}$	0.515	9.20

Table 5. Thermodynamic values of solubility products, respective B values and solubilities.

	$pS_{1,0}$	B	$pS_{2,0}$	B	pMA_0	Add. electrolyte
Ba	8.75	0.270	7.76	0.133	5.18	KCl
Sr	9.42	0.350	7.86	0.257	5.79	KCl
Ca	9.54	0.229	9.49	0.410	7.23	KCl
Mg			6.50	0.011	3.22	KCl
Mn			8.11	0.242	3.35	KCl
Cd			8.90	0.390	3.73	KCl
Zn			9.08	0.364	3.16	$NaNO_3$
Pb			10.35	0.3	4.39	$NaNO_3$
Co			8.96	0.510	2.90	$NaNO_3$
Ni			9.66	0.318	2.61	$NaNO_3$
Cu			11.87	0.410	3.01	$NaNO_3$

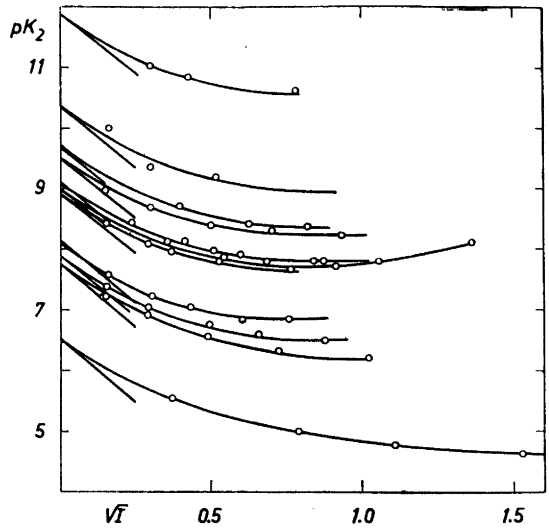

Fig. 5. Solubility products $[M^{++}][A^{=}]$ as functions of ionic strength. From below: Mg, Ba, Sr, Mn, Cd, Co, Zn, Ca, Ni, Pb, Cu.

tion was obtained by the first spectrophotometric method. Then using the measured pH value of the equilibrium solution and the ionization constant of the reagent, the concentration of $A^{=}$ was obtained. Also the concentration of lead ion in the solution can now be calculated by subtracting the sum of MA in the solution and the precipitate from the total lead concentration. The values obtained by this procedure are presented in Table 4 and are seen to be in a relatively good agreement with the results in Table 3.

The results on the solubility products can be presented by the equations

$$pS_1 = pS_{1,0} - \frac{3.05\sqrt{I}}{1 + 1.5\sqrt{I}} + BI \qquad (3)$$

and

$$pS_2 = pS_{2,0} - \frac{4.07\sqrt{I}}{1 + 1.5\sqrt{I}} + BI \qquad (4)$$

The values of $pS_{1,0}$, $pS_{2,0}$ and corresponding values of B are presented in Table 5. In Figs. 4 and 5 the solubility products are represented as functions of the ionic strength. We see from these figures that the effect of added electrolytes on the solubility products is fairly normal.

From equation (2) and from equation

$$[MA]/[M^{++}]\,[A^{=}] = k_1 \qquad (5)$$

we obtain

$$[MA] = k_1 S_2 \qquad (6)$$

In Table 5 the concentration of MA at zero ionic strength ($[MA]_0$) is also given. In the case of 8-quinolinol-5-sulphonic acid this quantity was about 10^{-3} for

most of the metals investigated. Only the chelates of barium, strontium, calcium and lead were less soluble. In the present case the state of things is not very much different except that the barium, strontium and calcium chelates are now about 10^2—10^3 times less soluble. Therefore the determination of the stability constants of the chelates of the last mentioned metals were carried out in supersaturated solutions [1]. This proved to be possible owing to the rather strong tendency of these metal chelates to supersaturation as mentioned above.

The stability constants of the metal chelates of 8-quinolinol derivatives, studied by us up to now, increase with increasing second ionization potential of gaseous metal atoms as in many other systems [7]. Further the respective solubility products follow the same order. On account of their exceptionally low solubility, the barium, strontium and calcium chelates do not follow this rule in the present case.

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Short Communications

Oxidation of Cerium(III) to Cerium(IV) Ion by Means of Cobalt(III) Ion

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Cobaltous carbonate, suspended in a solution of sodium or potassium bicarbonate, is oxidized by hydrogen peroxide to a green soluble cobaltic complex [1]. Job [2] and several other authors have shown — by measuring the oxidative power — that cobalt is trivalent in the green solution. Reduction of the green trivalent cobalt compound may be carried out by addition of excess of standard ferrous pyrophosphate and titration of the excess with permanganate [2,3]. Metzl [4] has determined cobalt volumetrically by first converting it into the green cobaltic complex. After addition of sodium hydroxide and boiling, all cobalt is precipitated as cobaltic hydroxide, which can be dissolved in sulfuric acid containing potassium iodide. The iodine formed is then titrated with sodium thiosulphate.

From an analytical point of view the green cobaltic carbonato ion (it is reasonable to assume that the green cobaltic complex is either $[Co(CO_3)_3]^{---}$ or mixed carbonato hydroxo ions) may act as a source of cobaltic aquo ion. When the green solution is acidified with a considerable excess of sulfuric or nitric acid, the blue cobaltic aquo ion is formed. Cobaltic ion is one of the most powerful oxidants known; the reduction potential of Co(III)/Co(II) is about 1.8 volts. However, solutions containing cobaltic aquo ion are not stable, because water acts as a reducing agent. The stability seems to increase with increasing acid concentration. — In the present preliminary investigation it is shown that cerous ion can be oxidized quantitatively to ceric ion by means of cobaltic ion.

Preparation of the cobaltic carbonato solution. 0.01 mole cobaltous nitrate was dissolved in 250 ml water. 75 g sodium bicarbonate was added and the flask agitated for one minute. Thereafter 2 ml 30 % hydrogen peroxide, diluted with water to 250 ml; were added gradually while rotating the contents of the flask. After dilution to 1 liter the still undissolved sodium bicarbonate went into solution. The clear green liquid was left until next day to make sure that the excess of hydrogen peroxide was quite destroyed. Fortunately the complex cobaltic ion exerts a strong catalytic influence on the decomposition of hydrogen peroxide, so, in all likelihood, the excess is destroyed within a few minutes.

The normality of the cobaltic solution was determined as follows: 50 ml of the green solution was run into 30 ml 2 *M* sulfuric acid plus 2 g potassium iodide. The iodine formed was then titrated with thiosulphate. The results were very reproducible. The carbon dioxide formed by the neutralization seems to expel the oxygen from the solutions. Anyhow, aeration of the solutions with carbon dioxide does not alter the titer. To make sure that the titer thus found really is identical with the content of cobalt, the cobalt was determined by electrolysis. The average normality found by titration was 0.00938. Two electrolytic determinations gave molarity with respect to cobalt 0.00936 and 0.00938.

It seems impossible to make the cobaltic carbonato solution much more than 1/100 molar even if the solution is saturated with sodium bicarbonate. The excess of cobalt will yield a brown precipitate. The *ca.* 1/100 molar solution keeps its titer fairly well, but when stored for months the titer

decreases considerably with precipitation of brown hydroxide. It seems that the solution remains more constant when sodium bicarbonate is present in excess (solid salt on the bottom of the container).

Oxidation of cerous ion. A comparison of the reduction potential of Co(III)/Co(II), which is about 1.8 volts, with that of Ce(IV)/Ce(III), which is about 1.5 volts, shows that it is theoretically just possible to oxidize cerous ion quantitatively by means of cobaltic ion in the usual analytical concentration range. The following experiments confirm this.

An about 0.05 M cerous nitrate solution was prepared and analyzed. Evaporation of a sample and ignition to CeO_2 gave the molarity 0.0515 (mean of 3 determinations). The colour of the CeO_2 showed that it was not quite pure, but with a slight content of other rare earths. The true content of cerous ion in the solution was determined by oxidation with sodium bismuthate [5,6] and titration with hydrogen peroxide of the Ce(IV) formed. Two determinations gave 0.0511 M and 0.0511 M.

The cerium content in 20 ml of the 0.0511 M solution was now oxidized with cobaltic ion as follows: 30 ml conc. sulfuric acid were diluted with water to 100 ml. After cooling 20 ml cerous nitrate solution were added. 150 ml *ca.* 0.01 M cobaltic carbonato solution were now gradually added. The Ce(III) was oxidized nearly immediately, and the colour of the solution showed excess of cobaltic ion. The solution was now heated to decompose the cobaltic ion, and boiled for one minute, to expel the oxygen formed. The ceric ion formed was now titrated with hydrogen peroxide. Two determinations gave 0.0509 M and 0.0510 M.

Cobaltic ion may be used for many other quantitative oxidations, for example oxidation of chromic chromium to chromate chromium.

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Separation of Saturated Straight Chain Fatty Acids II. Qualitative Paper Ionophoresis

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When analysing the homologous series of saturated straight chain fatty acids the separation can be achieved by paper ionophoresis. Barnett [1,2] describes the separation of long chain fatty acids — from capric to stearic acid — using 3 N aqueous ammonia as solvent. The analysis is, however, difficult to carry out because of the high volatility of ammonia. In the procedures described below this difficulty is avoided by substituting ammonia with sodium hydroxide.

The acids from acetic to pelargonic acid can be separated from each other ionophoretically by means of aqueous sodium hydroxide buffered with boric acid to pH 9.0. The mobilities of these acids at +25° C and at a voltage of 5 V/cm were as follows: acetic 31, propionic 27, butyric 25, valeric 23, caproic (hexoic) 21.5, önanthylic (heptoic) 20, caprylic (octoic) 19, and pelargonic (nonoic) acid 18 mm/h.

The acids from acetic to palmitic acid can be separated from each other ionophoretically by using a 0.2 N solution of sodium hydroxide in glycerol and a temperature of +90° C. The short chain acids are separable with the method described below but their location on the glycerolic paper is rather cumbersome because of the slow absorption of the indicator solution and because of the disturbing effect of the atmospheric carbon dioxide. Therefore the description will be limited to the separation of the acids from caproic acid upwards. As an interesting detail may be mentioned that it is possible to follow the movement of all these acids throughout the analysis by dissolving a suitable indicator, *e. g.* phenolphtalein, in the glycerol solution and removing the carbon dioxide from the air-tight reaction chamber equipped with a window. The mobilities of different acids at a voltage of 20 V/cm were as follows: caproic 7.7, önanthylic 6.6, caprylic 5.8, pelargonic 5.3, capric (decoic) 4.9,

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undecoic 4.6, lauric (dodecoic) 4.4, myristic (tetradecoic) 4.1, and palmitic (hexadecoic) acid 3.9 mm/h.

Experimental: Separation of the acids from acetic to pelargonic acid. The paper was used as 10 × 50 cm strips, each for the investigation of three samples. The distance between electrode vessels was 40 cm from liquid to liquid. The acids were placed on the paper as free acids in ethanol as close to the buffer solution in the cathode vessel as possible. In order to avoid the volatilization of the acids they were neutralized on the paper with ammonia vapour. After the solvent had evaporated the whole paper was sprayed with buffer solution the excess of which was removed by two dry filter papers. The paper was then immediately placed between the electrode vessels and kept there horizontally by means of glass rods fastened to the ends of the paper. The whole apparatus was surrounded by saturated water vapour. After ionophoresis the paper was dried. The acids were located with methyl red. The acids from valeric to pelargonic acid can also be located [3] with a solution of rhodamin B.

Separation of the acids from caproic to palmitic acid. The paper strips were of the same size as above, but in some cases it was advantageous to use shorter strips than 50 cm in order to speed up the analysis without increasing the electric potential. The paper was immersed in 0.2 N glycerolic sodium hydroxide. The excess of glycerol was removed with the edge of a glass plate. The ethanol solutions of the acids were placed on the paper and the paper laid horizontally between the electrode vessels. After the paper had reached the temperature of the chamber (90° C) the current was put on. The acids were located by spraying the paper first with 2 % aqueous copper acetate, removing the glycerol after 10 min. with water, drying the paper, and spraying it finally with a 0.5 % solution of rhodamin B in water or in 0.05 N hydrochloric acid. On the dried paper the acids were located conveniently in ultraviolet light.

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The Effect of Nitrous Oxide on Nitrate Utilization by *Azotobacter vinelandii*

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Nitrous oxide competitively inhibits nitrogen fixation by *Azotobacter vinelandii* but exerts no effect on ammonia utilization [1-3]. Virtanen and Lundbom [4] have found in their long time growth experiments that N_2O inhibits not only the fixation process but also nitrate reduction by this organism. Because of the importance of this finding for the understanding of the mechanism of biological nitrogen fixation, the effect of N_2O on nitrate utilization by *A. vinelandii* was reinvestigated in short time respiration experiments.

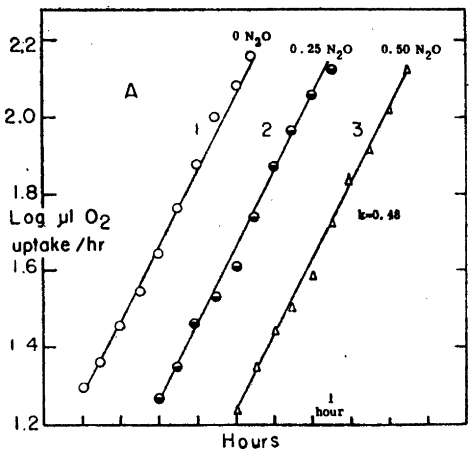

Fig. 1. Lack of inhibition by N_2O of growth of A. vinelandii (strain O) supplied nitrate. Growth was measured by following manometrically the increasing rate of oxygen uptake by the culture.

* Fellow of the John Simon Guggenheim Memorial Foundation.

undecoic 4.6, lauric (dodecoic) 4.4, myristic (tetradecoic) 4.1, and palmitic (hexadecoic) acid 3.9 mm/h.

Experimental: Separation of the acids from acetic to pelargonic acid. The paper was used as 10 × 50 cm strips, each for the investigation of three samples. The distance between electrode vessels was 40 cm from liquid to liquid. The acids were placed on the paper as free acids in ethanol as close to the buffer solution in the cathode vessel as possible. In order to avoid the volatilization of the acids they were neutralized on the paper with ammonia vapour. After the solvent had evaporated the whole paper was sprayed with buffer solution the excess of which was removed by two dry filter papers. The paper was then immediately placed between the electrode vessels and kept there horizontally by means of glass rods fastened to the ends of the paper. The whole apparatus was surrounded by saturated water vapour. After ionophoresis the paper was dried. The acids were located with methyl red. The acids from valeric to pelargonic acid can also be located [3] with a solution of rhodamin B.

Separation of the acids from caproic to palmitic acid. The paper strips were of the same size as above, but in some cases it was advantageous to use shorter strips than 50 cm in order to speed up the analysis without increasing the electric potential. The paper was immersed in 0.2 N glycerolic sodium hydroxide. The excess of glycerol was removed with the edge of a glass plate. The ethanol solutions of the acids were placed on the paper and the paper laid horizontally between the electrode vessels. After the paper had reached the temperature of the chamber (90° C) the current was put on. The acids were located by spraying the paper first with 2 % aqueous copper acetate, removing the glycerol after 10 min. with water, drying the paper, and spraying it finally with a 0.5 % solution of rhodamin B in water or in 0.05 N hydrochloric acid. On the dried paper the acids were located conveniently in ultraviolet light.

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The Effect of Nitrous Oxide on Nitrate Utilization by *Azotobacter vinelandii*

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Nitrous oxide competitively inhibits nitrogen fixation by *Azotobacter vinelandii* but exerts no effect on ammonia utilization [1-3]. Virtanen and Lundbom [4] have found in their long time growth experiments that N_2O inhibits not only the fixation process but also nitrate reduction by this organism. Because of the importance of this finding for the understanding of the mechanism of biological nitrogen fixation, the effect of N_2O on nitrate utilization by *A. vinelandii* was reinvestigated in short time respiration experiments.

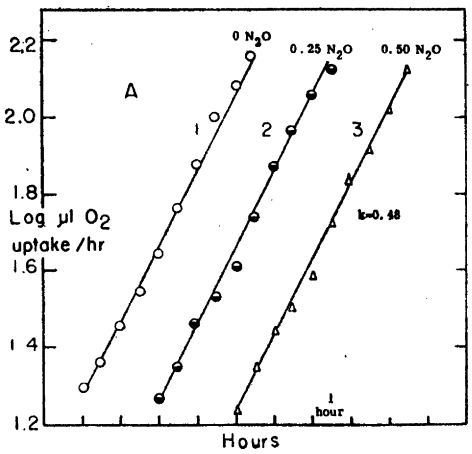

Fig. 1. Lack of inhibition by N_2O of growth of A. vinelandii (strain O) supplied nitrate. Growth was measured by following manometrically the increasing rate of oxygen uptake by the culture.

* Fellow of the John Simon Guggenheim Memorial Foundation.

Initial trials to demonstrate N_2O inhibition of nitrate assimilation were performed with *A. vinelandii* (Wisconsin strain O) which had been adapted by growth on nitrate; the growth of aliquots of the diluted culture was followed by measuring their increasing rates of respiration in Warburg respirometers. The respiration of cells incubated in Burk's medium containing 5 ppm NO_3^--N at 30° under 0.2 atm. O_2, and 0.0, 0.25 or 0.5 atm. N_2O (the diluent gas was He) is indicated in Fig. 1. The monomolecular rate constant for nitrogen assimilation, k, was about 0.48 for all the cultures; this is equivalent to a generation time of 1.6 hours. The effect of an inhibitor on such a vigorous culture should be detected readily; however, there was no significant inhibition of nitrate utilization even with 0.5 atm. N_2O.

Further experiments were conducted to test whether N_2O would affect the incorporation of ^{15}N from $^{15}NO_3^-$ into cells of *A. vinelandii*. Cells adapted to nitrate were supplied $^{15}NO_3^-$ (200 ppm N) and an atmosphere of 0.2 O_2 and either 0.8 N_2O or He. After the $^{15}NO_3$ had equilibrated with the normal nitrate of the medium, the isotope concentration in the nitrate was 5.11 atom per cent ^{15}N excess. The effect of N_2O on $^{15}N_2$ fixation also was examined to serve as a control and to confirm by an independent method the previously reported inhibition[1,2]. Cells which had been fixing N_2 were provided 0.1 atm. $^{15}N_2$ (6.95 atom per cent ^{15}N excess), 0.2 atm. O_2, and either 0.7 atm. N_2O or He. At intervals after initiation of incubation at 30°, the cells were sampled for ^{15}N analysis. The data in Table 1 show no inhibition of nitrate utilization by N_2O beyond the error of measurement, whereas over 80 % inhibition of N_2 fixation by N_2O is indicated.

Table 1. Uptake of ^{15}N by A. vinelandii (strain O) from labeled N_2 and nitrate as influenced by nitrous oxide.

Incubation time, hours	Atom per cent ^{15}N excess in cells			
	$^{15}NO_3^-$ supplied		$^{15}N_2$ supplied	
	0.8 N_2O	0.8 He	0.7 N_2O	0.7 He
1			0.074	0.442
			0.068	0.597
2	0.655	0.591		
	0.494	0.760		
3			0.287	1.463
			0.202	1.653
4	1.565	1.867		
	1.827	1.230		
5			0.475	2.073
			0.471	

Table 2. The effect of nitrous oxide on nitrogen assimilation by A. vinelandii (Jensen culture K).

Incubation time, hours	Mg N in cells		Atom per cent ^{15}N excess in cells			
	N_2 supplied		$^{15}NH_4^+$ supplied		$^{15}NO_3^-$ supplied	
	0.6 N_2O	0.6 vac.	0.6 N_2O	0.6 vac.	0.6 N_2O	0.6 vac.
3	0.124	0.185	1.39	0.816	0.131	0.113
	0.128	0.151	0.871	0.841	0.144	0.121
8	0.197	0.182	1.29	1.22	0.150	0.243
	0.197	0.144	1.28	1.85	0.183	0.483
19	0.239	0.422	2.39	2.78	0.433	0.391
	0.207	0.290	2.87	2.64	0.417	0.468
24	0.233	0.533	3.73	3.70	0.858	0.877
		0.412	4.08	3.94	1.01	0.842

Confirmation of these results was provided by experiments conducted in Helsinki with Jensen's culture K of *A. vinelandii*, the same strain of bacteria employed in the original study by Virtanen and Lundbom [4]. Cultures were supplied nitrogen either as 0.2 atm. normal N_2, 50 ppm ammonium nitrogen (7 atom per cent ^{15}N excess), or 500 ppm nitrate nitrogen (7 atom per cent ^{15}N excess). The test flasks were incubated in desiccators at 30° under 0.2 atm. N_2, 0.2 atm. O_2, and either 0.6 atm. N_2O or 0.6 atm. vacuum. The cells were harvested at 3, 8, 19, and 24 hours after inoculation. Total cellular nitrogen was determined, and the cells exposed to $^{15}NH_4^+$ or $^{15}NO_3^-$ also were analyzed for ^{15}N. The results of these experiments are summarized in Table 2; the results for total nitrogen on cultures furnished $^{15}NH_4^+$ and $^{15}NO_3$ are not recorded in the table, but they gave a picture entirely comparable to the ^{15}N analyses listed. These data further substantiate the specificity of N_2O inhibition for nitrogen fixation. It is clear that *A. vinelandii* using combined nitrogen either as ammonia or nitrate, is not inhibited by nitrous oxide.

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Studies on the Chemistry of Lichens. I. D-Arabitol from *Alectoria jubata* Ach., var. *chalybeiformis* Th.Fr.

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In the course of a series of investigations on lichen substances in the genus *Alectoria*, the *Alectoria jubata* var. *chalybeiformis* has been studied.

Only one lichen substance, D-arabitol, was obtained by extraction of the lichen with acetone. It crystallized very slowly from saturated solutions. Occasionally the substance precipitated as an oily product from acetone or alcohol and solidified to a crystalline mass on standing or by scratching.

An authentic sample of D-arabitol was not available. The specific rotation from saturated, aqueous boric acid solution was +7.66°, in fair agreement with the value of +7.82° reported by Asahina [1].

X-ray diffraction patterns of the substance and of L-arabitol were identical.

Experimental. Air-dried, ground *Alectoria jubata* var. *chalybeiformis* (208 g) (collected by cand.real. Eilif Dahl, on birch in Rondane, Norway) was continuously extracted with acetone for twenty hours.

The greater part of the solvent was removed by distillation, and on standing in a refrigerator the solution deposited colourless crystals (A). These were separated, washed with ether and dried. The dark green filtrate was concentrated to a small volume and the viscous residue repeatedly treated with cold alcohol. Undissolved material was removed by filtration (B). A and B both appeared to be the same substance and were purified together by recrystallization from acetone. After having been kept at —22° for some ten days 0.32 g of

Table 1. Spacing values in Å. (w = weak, m = medium, s = strong, vs = very strong.)

Substance from *Alectoria jubata* var. *chalybeiformis*	L-arabitol
7.37 s	7.37 s
5.48 »	5.48 »
4.83 vs	4.83 vs
4.63 »	4.60 »
4.33 »	4.33 »
3.96 w	3.95 w
3.60 vs	3.61 vs
3.42 s	3.42 s
3.23 »	3.23 »
3.12 »	3.11 »
2.83 m	2.83 m
2.52 s	2.52 s
2.41 w	2.42 w
2.37 »	2.38 »
2.15 s	2.16 s
2.08 »	2.09 »
2.03 w	2.03 w

Confirmation of these results was provided by experiments conducted in Helsinki with Jensen's culture K of *A. vinelandii*, the same strain of bacteria employed in the original study by Virtanen and Lundbom [4]. Cultures were supplied nitrogen either as 0.2 atm. normal N_2, 50 ppm ammonium nitrogen (7 atom per cent ^{15}N excess), or 500 ppm nitrate nitrogen (7 atom per cent ^{15}N excess). The test flasks were incubated in desiccators at 30° under 0.2 atm. N_2, 0.2 atm. O_2, and either 0.6 atm. N_2O or 0.6 atm. vacuum. The cells were harvested at 3, 8, 19, and 24 hours after inoculation. Total cellular nitrogen was determined, and the cells exposed to $^{15}NH_4^+$ or $^{15}NO_3^-$ also were analyzed for ^{15}N. The results of these experiments are summarized in Table 2; the results for total nitrogen on cultures furnished $^{15}NH_4^+$ and $^{15}NO_3$ are not recorded in the table, but they gave a picture entirely comparable to the ^{15}N analyses listed. These data further substantiate the specificity of N_2O inhibition for nitrogen fixation. It is clear that *A. vinelandii* using combined nitrogen either as ammonia or nitrate, is not inhibited by nitrous oxide.

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the substance was obtained. After washing with ether and recrystallization from ethanol-petroleum ether in 1:1 ratio 0.16 g of pure D-arabitol was obtained; m. p. 100° (uncorr.). (Found: C 39.54; H 8.06. Calc. for $C_5H_{12}O_5$: C 39.47; H 7.95.) $[\alpha]_D^{21}$ + 7.66° (from saturated, aqueous boric acid solution, c = 2.2).

The comparison of X-ray diffraction patterns of L-arabitol and the substance from *Alectoria jubata* var. *chalybeiformis* crystallized from acetone gave the spacing values in Å, recorded in Table 1. The instrument used was one Philips X-ray Geiger Counter Spectrometer with Cu-$K\alpha_1$ radiation.

L-Arabitol was kindly provided by Farmasöytisk Institutt, Blindern-Oslo, Norway.

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Sialic Acid in Human Cervical Mucus, in Hog Seminal Gel, and in Ovomucin

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Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

The presence of sialic acid as a characteristic constituent in epithelial mucus has been indicated by colour reactions [1]. This and/or closely related substances have also been isolated in crystalline form from certain materials of epithelial origin [2-8]. The isolation of a sialic acid from three additional similar sources, *viz.* human cervical mucus, the gelatinous lumps of hog semen, and ovomucin, will be reported here.

Using quantitative colorimetric methods Werner [1] has found, that the mucous plugs of the pregnant human uterine cervix contain considerable amounts of sialic acid in addition to glycoprotein material of the same general composition as the blood-group substances.

The carbohydrate components of ovomucin, the probably non-homogeneous, water-insoluble glycoprotein of hen's eggs, have been reported to be galactose and mannose in approximately equimolecular amounts [9], together with glucosamine and galactosamine [10]. By means of colour reactions the presence in ovomucin of fairly large amounts of substance of the sialic acid type has been indicated [10,11].

In hog semen a voluminous gel is formed shortly after ejaculation. No recent investigations on the composition of this material seem to have been published. From human seminal plasma Freudenberg *et al.*[12] have prepared a product showing the properties of the blood group substances. The preparation from the same material of an electrophoretically homogeneous glycoprotein, containing hexosamine but not uronic acid, has also been reported [13].

Experimental. Analytical methods. The same methods as those recently reported [8] were used. Separation of the aminosugars was performed by Gardell's method [14]. The single monosaccharide components in the glycoprotein hydrolysates were determined after paper-chromatographic separation. The X-ray powder diagrams were taken with a Guinier camera using copper $K\alpha$-radiation.

Materials. The cervical mucous plugs were collected immediately before delivery, and stored in 60 % aqueous ethanol. When enough material was obtained the plugs were cut up with scissors and kept in about 0.005 N hydrochloric acid for 24 hours at 4° C. After washing with aqueous ethanol the material was dried with ethanol and ether. The dry powder contained 9.7 % nitrogen, 6.6 % glucosamine, 3.9 % galactosamine, 7.5 % galactose, 5.0 % fucose, and 7.4 % sialic acid.

The colourless gelatinous mass from two ejaculates from a boar was thoroughly washed with saline and distilled water after decanting off the fluid seminal plasma. It was then cut in small pieces, treated with hydrochloric acid, and dried exactly as the cervical mucus. The analysis gave the following composition: 10.7% nitrogen, 2.0 % glucosamine, 8.5 % galactosamine, 2.7 % galactose, 1.3 % mannose, 0.7 % fucose, and 14.7 % sialic acid.

The ovomucin was prepared mainly according to the method of Gottschalk and Lind [15]. To cold homogenized egg-white, freed from chalazae by passing through a filtering cloth, cold distilled water was added. The mixture was centrifuged, and the gelatinous precipitate obtained dispersed in 10 % sodium chloride and reprecipitated by addition of distilled water. The precipitate was washed with saline and distilled water until the washings were free from protein. The material was then treated with hydrochloric acid, and dried in the

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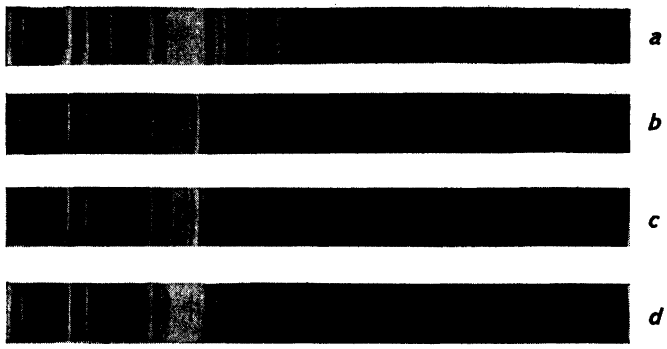

Fig. 1. X-ray powder diagrams of sialic acid isolated from (a) a human ovarian cyst gel, (b) human cervical mucus, (c) hog seminal gel, and (d) ovomucin.

usual way. The dry powder had the following composition: 13.3 % nitrogen, 4.7 % glucosamine, 2.4 % galactosamine, 4.4 % galactose, 2.2 % mannose, and 6.0 % sialic acid.

The *isolation of sialic acid* from these materials was performed according to the principles set out by Blix [2]. As only minute amounts of crystalline material were obtained from ovomucin by heating with water, weak sulphuric acid was used for the hydrolysis, but the yields remained small.

The dry powders from the cervical mucus (2 g) and hog seminal gel (10 g) were heated with distilled water on a boiling water-bath under reflux for one hour. The viscous fluids containing much undissolved gelatinous material were freeze-dried.

The ovomucin preparation (10 g) was heated with weak sulphuric acid (pH 2—3) in the same way. The hydrolysate was centrifuged. To the filtered supernatant barium hydroxide was added to pH about 8. After filtering, the solution was passed through a cation exchange column (Amberlite IRC-50, H), and then freeze-dried.

Crystalline products were obtained from the dried materials by the procedure recently described [3]. The crystallization appeared to be promoted by placing the specimens alternately in the refrigerator and at room temperature. From the cervical mucus about 30 mg of crystalline material was obtained, from the seminal gel about 150 mg, and from the ovomucin about 15 mg.

The X-ray diffraction patterns of the crystalline substances isolated from these three sources were all identical (Fig. 1). They were also identical with that of the sialic acid isolated from sheep submaxillary mucin by Blix *et al.*[2] and from some human sources by Odin [7,8]. Quantitative determinations with the Bial and Ehrlich reagents gave also the same extinction values for all the substances.

Identical sialic acids have thus been isolated from ovine, human, porcine, and avian sources. This substance is probably the naturally occurring sialic acid in these materials. Some changes in the molecule of an original substance occurring during the isolation procedure cannot, however, be excluded. It should be noted that the sialic acid, which has been isolated from hog submaxillary mucin [3], is not identical with the substance obtained from hog semen in the present investigation.

I wish to express my gratitude to Professor J. Naeslund, Dept. of Obstetrics and Gynaecology, University of Uppsala, and his nursing staff, and to Dr. I. Settergren, Royal Veterinary College, Stockholm, for material used in this study. I also wish to thank Professor G. Hägg, Chemical Institute, University of Uppsala, for allowing me to take the X-ray photographs in his laboratory.

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Preparation of 2,5-Dimethyl-1,4-Dioxan

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In a recent paper it has been shown that Levene and Walti's method for the preparation of 2,6-dimethyl-1,4-dioxan yielded the cyclic acetal 2-ethyl-4-methyl-1,3-dioxolan [1]. 2,6-Dimethyl-1,4-dioxan, however, has been prepared by Nesmeyanov and Lutsenko [2]. In the course of studies related to addition compounds of ethers, which are being carried out in this laboratory it was found desirable to prepare 2,5-dimethyl-1,4-dioxan as well. Only one previous work is reported on the preparation of this compound. I. G. Farbenindustrie in a patent states that the compound is formed when propylene oxide vapours are passed over sodium bisulphate or other acidic substances at elevated temperatures [3]. The sodium bisulphate reaction, however, when repeated here was found to give a mixture of compounds.

Looking for another starting material for the preparation of pure 2,5-dimethyl-1,4-dioxan we discovered that *trans*-2,5-bis-(iodomethyl)-1,4-dioxan has been prepared through a few intermediates from allyl alcohol and mercuric nitrate [4]. This compound should be expected to give 2,5-dimethyl-1,4-dioxan on reduction.

However, experiments showed that the dioxan ring may be subject to easy cleavage under reducing conditions. By the use of zinc and ethanol or zinc and acetic acid as reducing agents allyl alcohol or allyl acetate were formed, respectively. This difficulty was overcome by using lithium aluminium hydride, which yielded the desired product. Physical constants of the purified reaction product: B.p. (750 mm) 121.5°; m. p. −4.5°; mol.wt. 113.9 (calc. for dimethyldioxan 116.2); d_4^{22} 0.932; n_D^{22} 1.4147; mol. refraction 30.61 (calc. 30.99).

1,4-Dioxan is known to form addition compounds with numerous inorganic salts. An X-ray structure investigation of the mercuric chloride addition compound has been carried out by Hassel and Hvoslef [5].

The 2,5-dimethyldioxan prepared in the present work forms a similar addition compound with mercuric chloride. An X-ray structure investigation of this addition compound has been started in order to confirm that the methyl groups are mutually "*trans*" situated.

Experimental. To 63 g of *trans*-2,5-bis-(iodomethyl)-1,4-dioxan prepared according to Summberbell and Stephens [4] were added 1 l of ether and 20.5 g of lithium aluminium hydride. The round bottom flask was equipped with a sealed stirrer and reflux condenser with a drying tube on top. The stirring was continued with gentle boiling for 8 days. The actual reaction time is probably shorter, but in a preliminary test experiment unreacted starting material was recovered after several days. Finally water was added drop by drop until the evolution of hydrogen had ceased. The etheral layer was separated from the precipitate, the latter washed several times with ether and the ether solutions combined. The main part of the ether was removed by distillation, rest *ca.* 200 ml. To remove iodine the solution was shaken until it became colourless with a few ml of a saturated aqueous sodium thiosulphate solution. After drying over anhydrous sodium sulphate the ether was distilled off and the remaining liquid distilled in a 24 inches Podbielniak column with a reflux ratio of 50 : 1. The liquid distilled at constant temperature .Yield *ca.* 15 ml. (Found: C 61.89; H 10.34. Calc. for $C_6H_{12}O_2$: C 62.01; H 10.41).

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Preparation of 2,5-Dimethyl-1,4-Dioxan

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In a recent paper it has been shown that Levene and Walti's method for the preparation of 2,6-dimethyl-1,4-dioxan yielded the cyclic acetal 2-ethyl-4-methyl-1,3-dioxolan [1]. 2,6-Dimethyl-1,4-dioxan, however, has been prepared by Nesmeyanov and Lutsenko [2]. In the course of studies related to addition compounds of ethers, which are being carried out in this laboratory it was found desirable to prepare 2,5-dimethyl-1,4-dioxan as well. Only one previous work is reported on the preparation of this compound. I. G. Farbenindustrie in a patent states that the compound is formed when propylene oxide vapours are passed over sodium bisulphate or other acidic substances at elevated temperatures [3]. The sodium bisulphate reaction, however, when repeated here was found to give a mixture of compounds.

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Analysis of Steroids in Dog Adrenal Vein Blood by Countercurrent Distribution

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Besides Δ^4-pregnene-11β, 17a, 21-triol-3,20-dione (cortisol) and Δ^4-pregnene-11β, 21-diol-3,20-dione (corticosterone) three new, very nonpolar ketonic steroids have been demonstrated in appreciable amounts in adrenal vein blood of dogs, two of them being Δ^4-3-keto-steroids and one of these having at least one hydroxyl group.

The separation of pure corticosteroids by countercurrent distribution has been reported elsewhere [1]. Adrenal vein blood was collected from anaesthetized dogs held at constant blood pressure. The blood was cooled during the collection and thereafter frozen to −20° C. After rapid thawing it was diluted with distilled water (1:1) and extracted with ethyl acetate by carrying out a fine droplet distribution of the blood into the extracting medium. The dried extract was distributed twice between *n*-hexane and 50 % ethanol in water. The combined lower phase is expected to contain all conventional corticosteroids possibly present, since their partition coefficients are less than 0.09 in this system. Different components with specific extinction at 240 mμ were separated by countercurrent distribution using 24 transfers and a sequence of different solvent systems (Table 1). The upper phase of the original distribution was subjected to a micro-Girard separation [2]. Countercurrent distribution of the ketonic fraction (Table 2) revealed the presence of two compounds with extinction maximum at 240 mμ (designed x and z). The x-component was acylable as the partition coefficient shifted from 0.67 to 6.7 in the same system after treatment with acetyl chloride (Table 2). The blue tetrazolium [3], Porter-Silber [4] and Zimmermann [5] reactions were negative. The last reaction was negative after oxidation with sodium bismuthate [6] as well. A third component (designed y) appeared having an extinction maximum between 276 and 280 mμ. The Kober [7], blue tetrazolium and Porter-Silber reactions were negative, but a sharp extinction peak appeared at 260—265 mμ with the phenylhydrazine-sulfuric acid reagent. This was

Table 1.

No.	Countercurrent distribution (CC) Solvent system	Followed by CC No.	Partition coefficients (K) of polar components in adrenal vein blood: K	*Steroid identified*
1:	Benzene/H_2O	2	0.35	
		3	11.5	
2:	20 % Ethanol + 80 % H_2O / 50 % $CHCl_3$ + 50 % *n*-hexane		2.57	Cortisol
3:	60 % *n*-Hexane + 40 % benzene	4	0.70	
	5 % Ethanol + 95 % H_2O	5	11.5	
4:	20 % Ethanol + 80 % H_2O / CCl_4		1.6	Corticosterone
5:	*n*-Hexane / H_2O		1.8	Not identified Only a small amount

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Table 2.

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	Pure compounds designed below			Components in adrenal vein blood			
	DC	P	Δ^4-A	x	x-Ac	y	z
n-Hexane / 50 % ethanol + 50 % H_2O	0.09	0.9		10			
n-Hexane / 75 % ethanol + 25 % H_2O			0.08	0.67	6.7		
n-Hexane / 90 % ethanol + 10 % H_2O				0.41		2.70	8.1

DC: Δ^4-Pregnene-21-ol-3,20-dione (Cortexone)
P: Δ^4-Pregnene-3,20-dione (Progesterone)
Δ^4-A: Δ^4-Androstene-3,17-dione
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Semimicro Synthesis of Dihydrouracil and Uracil Labeled in Position 4, 5 or 6 with Carbon^{14}C

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Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

Recent evidence indicates the dihydropyrimidines as intermediates in the catabolism of the pyrimidine bases. Lieberman *et al.*[1,2] have reported the isolation of an enzyme system from an anaerobic soil bacterium which reduced orotic acid to dihydroorotic acid. Di Carlo *et al.*[3] suggested dihydrouracil as an intermediate in uracil catabolism from studies on the assimilation of nitrogen by *Torula utilis*. The breakdown of dihydrouracil to β-alanine in rat liver slices is shown by Fink *et al.*[4], and Funk *et al.*[5] reported the isolation of dihydrouracil from beef spleen.

For further investigation of the metabolism of uracil and dihydrouracil and their biological relation, a semimicro synthesis of the two pyrimidines labeled in position 4, 5 or 6 has been worked out, using potassium cyanide-^{14}C or chloroacetic acid labeled with ^{14}C in the 2 or 1-position, respectively, as starting materials.

126 mg of potassium cyanide containing 2 mC of ^{14}C were treated with 244 mg of the potassium salt of chloroacetic acid, and the resultant potassium cyanoacetate was catalytically hydrogenated under a pressure of 2 atm. at 18° for 11 hours to β-alanine [6,7]. After two crystallizations from alcohol-water the crude β-alanine was reacted with 160 mg of potassium cyanate at 25°, forming the potassium salt of β-ureidopropionic acid [8,9]. Treatment of the crude product with hydrochloric acid and subsequent heating of the dry substance to 170° yielded 119 mg (56 % based on chloroacetic acid) of dihydrouracil-4-^{14}C crystallized from water. On recrystallization from water, adding charcoal, the product melted at 274—275° (reported [10] m. p. 275—276°). The theoretical specific activity was 9.1 μC per mg.

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40 mg of the dihydrouracil were brominated according to the method of Gabriel [11]. Subsequent heating of the crude product to 200° yielded 34.6 mg (88 % based on dihydrouracil) of uracil-4-^{14}C crystallized from water. On recrystallization from water, adding charcoal, the product melted at 335° as reported [10] for uracil. The theoretical specific activity was 9.1 μC per mg.

Attempts to prepare uracil by oxidation of dihydrouracil by alloxan according to Johnson [12] were unsuccessful.

Preparation of β-ureidopropionic acid labeled in the 1, 2 or 3-position is accomplished readily in aqueous solution according to Batt *et al.*[3] by decomposition in alkali of dihydrouracil labeled in position 6, 5 or 4 respectively.

The author wishes to thank Miss Jorunn Lassen for technical assistance in the synthesis of dihydrouracil.

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Combination of Unit Processes for Isolating Plutonium

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The various laboratory and industrial methods used in the isolation of plutonium from neutron irradiated uranium can be divided into a few unit processes, from which a relatively small number of simple flow-sheets can be derived. Some of these unit processes and flow-sheets are discussed.

When uranium has been irradiated for some time with slow neutrons it contains a certain amount of fission products, and a roughly equivalent amount (by weight) of plutonium. It is a difficult chemical problem to isolate this plutonium in the form of some pure compound and also to recover the uranium, whether this is done in the laboratory or on an industrial scale, since the plutonium and the uranium have to be separated from the fission products, which consist of about 30 different elements spread out over all groups in the periodic system. In addition, the fission products cause great health hazards because of their penetrating radiation, and should be removed almost completely from the plutonium and the uranium.

1. SURVEY OF COMBINED PROCESSES

Excellent reviews have been given of the chemistry of plutonium [1-3] as well as methods for its isolation from neutron irradiated uranium [4-6]. The aim of this paper is to point out how all these processes, published or unpublished, can be regarded in principle as composed of a number of unit processes, namely:

Reduction-oxidation processes, which we shall denote by (V for valency)

V66, giving Pu(VI) and U(VI),
V46, giving Pu(IV) and U(VI),
V36, giving Pu(III) and U(VI),
V34, giving Pu(III) and U(IV).

Table 1. Examples of media and treatments that will give plutonium and uranium in the valency states indicated. All time refers to plutonium concentrations $\leq 10^{-3}$ M.

Unit process V66: oxidation to Pu(VI)* and U(VI).

a. 70 % $HClO_4$, 195° C, 15 min [24].
b. 0.1 M $S_2O_8^{2-}$, 1 M H^+, 20° C, Ag^+ catalyst, 5 min.
c. 0.1 M $Cr_2O_7^{2-}$, 1 M H^+, Co^{2+} catalyst, 90° C, 10 min.
d. 0.2 M BrO_3^-, 1 M H^+, 95° C, 30 min.
e. $NaBiO_3$, 5 M H^+, 20° C, 5 min.

Unit process V46: transformation to Pu(IV)* and U(VI).

a. $\geq$8 M HNO_3, 90° C, 30 min.
b. conc. H_2SO_4, several hours fuming.
c. $\geq$8 M $HClO_4$, 90° C, 30 min.

Unit process V36: reduction to Pu(III)*, U(VI).

a. pass in SO_2, 1 M H^+, 20° C, 15 min.
b. 0.05 M N_2H_4, 2 M H^+, Fe^{2+} catalyst, 20° C, 20 min.

Unit process V34: reduction to Pu(III)* and U(IV).

a. Fe-powder, 1 M HCl, 20° C, 5 min.
b. Zn or Zn-Hg, pH$\leq$1, 20° C, 1 min.
c. electrolysis, 1 M H^+, 20° C *cf.* [26].
d. dissolving of metal in conc. HCl, F^- catalyst.
Note: After reduction a short aeration may be necessary to oxidize U(III) to U(IV).

* Mainly according to Ref.[1]

Separation processes, denoted by

S6, when Pu(VI) and U(VI) are collected together in a separate phase,
S46, when Pu(VI) or Pu(IV) and U(VI), or U(IV), are collected together in a separate phase,
S4, when Pu(IV) or U(IV) is collected in a separate phase,
S34, when Pu(III) and U(IV), or Pu(IV), are collected together in a separate phase,
S3, when Pu(III) is collected in a separate phase.

Tables 1 and 2 give examples of the principal reduction-oxidation and separation unit processes. They are also discussed in the following sections 2 and 3.

In each of the separation processes, some of the fission products will follow the actinides: the fission products accompaning the hexavalent, tetravalent and trivalent actinides will be denoted by FP^6, FP^4 and FP^3, respectively. The remaining fission products are denoted by FP^x.

Table 2. Examples of processes for the separation of actinides.

Unit process S6: separation of hexavalent actinides.

a. 0.5 M HNO_3, 2 M $Mg(NO_3)_2$, extraction with diethyl ether [4]
b. 0.1 M HNO_3, 3 M $Ca(NO_3)_2$, extraction with hexone [27]
c. pH 1.5, extraction with 3 M acetylacetone in hexone [28]
d. precipitation with 1 M CH_3COOH, 0.5 M CH_3COONa, 5 M $NaNO_3$, U(VI) carrier [4]

Unit process S46: joint separation of tetravalent and hexavalent actinides.

a. 8 M HNO_3, extraction with diethyl ether [29]
b. 1 M HNO_3, 4 M $Ca(NO_3)_2$, extraction with hexone [27]
c. pH 4, extraction with 3 M acetylacetone in $CHCl_3$ [28]
d. 2 M HNO_3, extraction with tributyl phosphate [7]
e. precipitation with excess of H_2O_2, pH ~1 [30]

Unit process S4: separation of tetravalent actinides.

a. pH 1.0, extraction with 1.5 M cupferron in $CHCl_3$ [31]
b. pH 1.0, extraction with 0.1 M thenoyltrifluoroacetone (TTA) in C_6H_6 [32,33]
c. pH 2.0, extraction with 2 M acetylacetone in C_6H_6 (only U(IV), Pu(IV)) [28]
d. precipitation with 0.2 M IO_3^-, 3 M H^+, Ce(IV) or Zr(IV) carrier [3,4,30]
e. precipitation with 0.3 M H_3PO_4, 3 M H^+, Zr(IV) or Bi(III) carr. [3,4,13,30]
f. precipitation with *m*-nitrobenzoic acid, pH ~3, Th(IV) carrier [30]

Unit process S34: joint separation of trivalent and tetravalent actinides.

a. precipitation with 1—3 M HF, 1 M H^+, La(III) carrier [3,4,30]
b. precipitation with 0.1 M $H_2C_2O_4$, 1 M H^+, Ce(IV) or Th(IV) carrier [3,4,30]
c. sorption on cation exchange resin, elution with complexing agent *e.g.*[10]

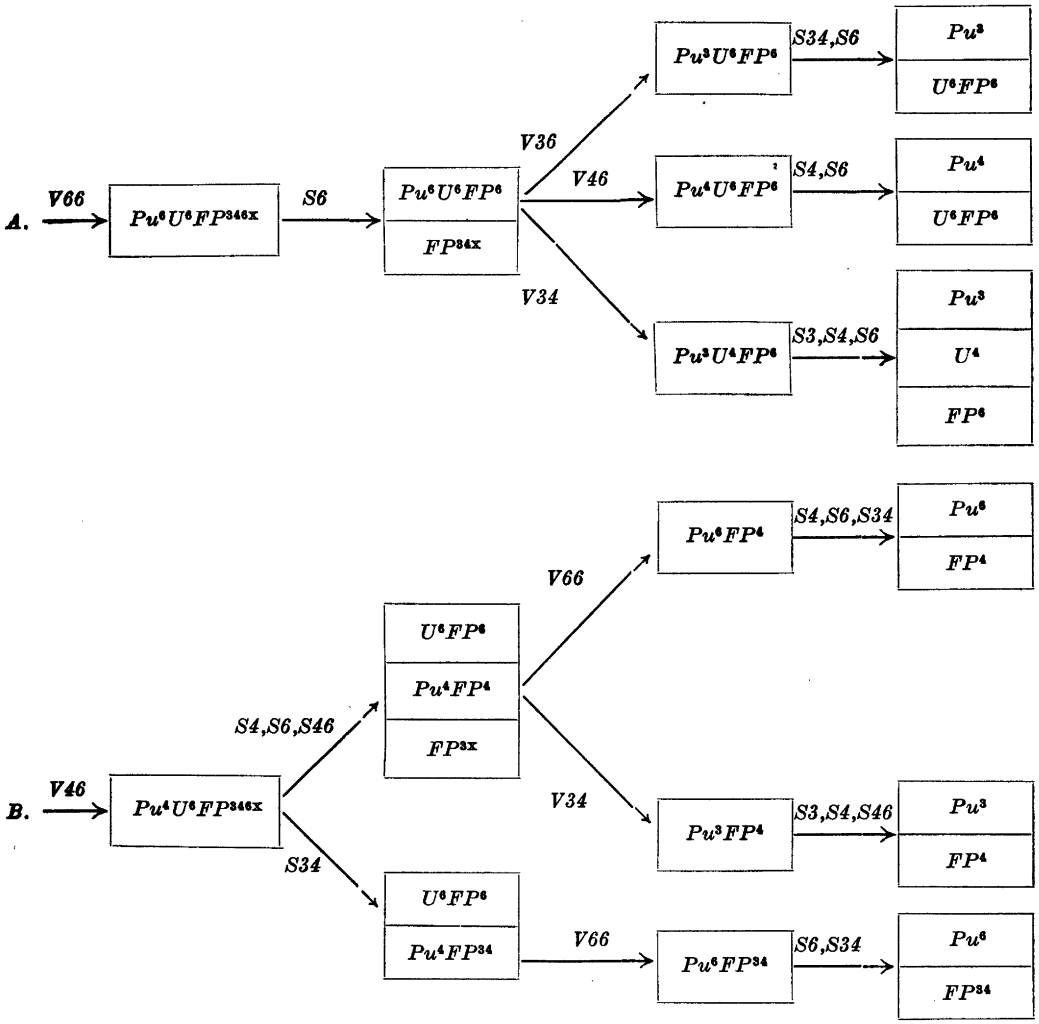
Unit process S3: separation of trivalent actinides.

No good process for a selective separation of trivalent actinides has been described. The best approximation to an S3 process may be an S34 process followed by an S4 or S46 process.

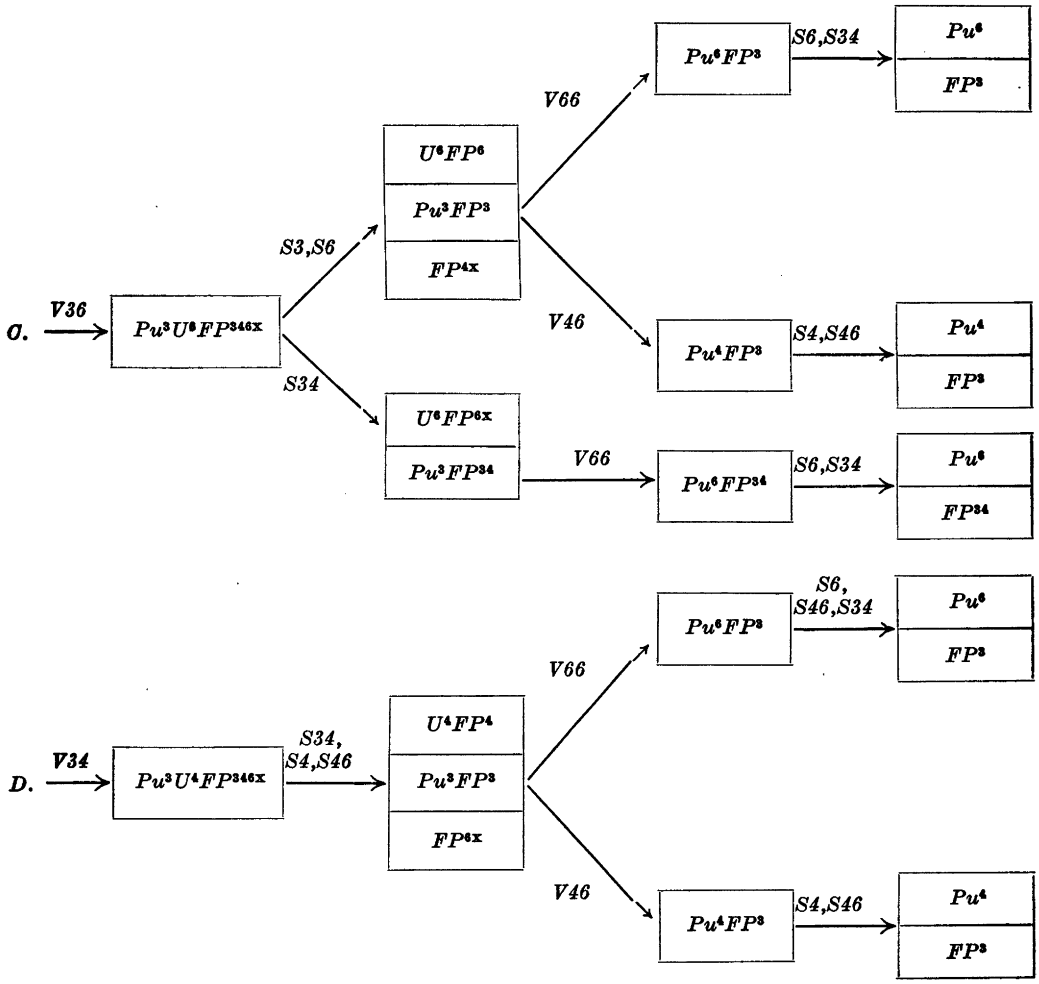
Table 3 gives a survey of the simplest combinations of the unit processes. The main interest has been in the isolation of plutonium. Most of the processes lead to uranium mixed with some fission products, generally FP [6]. This mixture can be separated by applying a suitable combination of unit processes, although this will not be discussed here.

It has been assumed in Table 3 that the fission products normally keep their oxidation numbers constant in the different processes. This is generally true, although important exceptions exist, which have to be considered in a more detailed discussion. Although most of the fission products may be removed by processes given in the diagrams, it may be necessary to add some extra operation for removing some particular fission products.

Table 3. Some possible combinations of unit processes for the isolation of plutonium from neutron irradiated uranium. The superscripts indicate valency states; the letter-figure combinations on the arrows refer to unit processes in Tables 1 and 2. See text for significance of single, double or triple rectangles.



The diagrams in Table 3 are self-explanatory. *A single rectangle* with its preceding arrow indicates the redox-process through which the different valency states are obtained. *Two adjacent rectangles* with the preceding arrow indicates a process in which the elements have been separated into two different phases (*e. g.* aqueous phase, precipitate, ion exchanger, organic solvent); any process on the arrow can be used. *Three adjacent rectangles* with the preceding arrow indicate the separation of the elements into at least three phases,

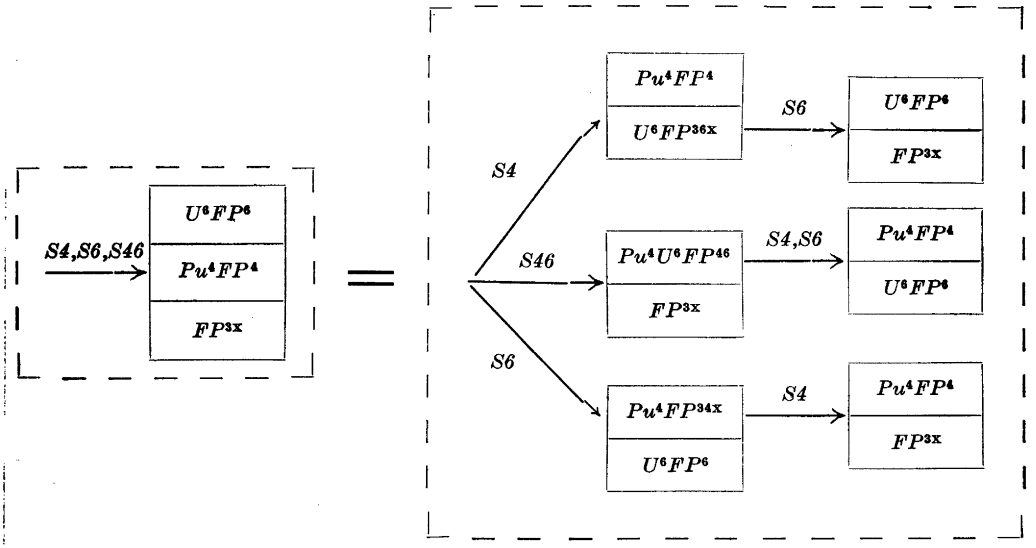


by two subsequent separation processes, which can be applied in an arbitrary order; at least two of the processes on the arrow have to be used. As an example of the last case, the second upper step of diagram B in Table 3 has been fully expanded in Table 4.

2. REDUCTION-OXIDATION PROCESSES

When irradiated uranium metal is dissolved in non-oxidizing agents, one will generally find the oxidation states Pu(III) and U(IV). When dissolved in hot HNO_3 the final products are mainly Pu(IV) and U(VI). A very large number of reduction-oxidation processes can be used to convert plutonium and uranium into other valency states.

Table 4. The possible combinations of a "three adjacent rectangles" unit process. Any combination of processes S4, S46 and S6 will yield the same result: Pu^4FP^4, U^6FP^6 and FP^{3x} in three different phases after two separation processes. The example is taken from diagram B in Table 3, the second step of the upper flow-sheet.



The time and temperature for a conversion >90 % for some processes in non-complexing media are given in Table 1. The processes are usually faster at lower H^+ concentrations.

In some of the V36-processes, U(VI) is also reduced, but the latter reduction is so slow, that it can be neglected during the time of reduction of plutonium (*e. g.* the reduction with SO_2 in V36a). Since the equilibrium between Pu(IV) and Pu(V, VI) is often attained very slowly, the reaction has to be catalysed.

Although tracer amounts of plutonium can be kept for quite a long time as Pu(VI) in non-reducing solutions, or as Pu(III) in non-oxidizing solutions, Pu (IV) disproportionates rapidly into Pu(III) and Pu(VI) at low acidities. Considerable amounts of Pu(V) may also be formed. However, the disproportionation is less than 1 % per hour at pH <1, and almost negligible at hydrogen ion concentrations above 5 M.

The presence of a complexing agent may stabilize a certain valency state. *E. g.*, if H_2SO_4 is used for adjusting pH, the Pu(IV) valency state will be stabilized, and the oxidation to Pu(VI) or reduction to Pu(III) will proceed much slower, if at all.

It must be stressed that when the oxidation states of plutonium or uranium are changed, some fission products also change their valency state. *E. g.*, when Pu(IV) is oxidized to Pu(VI), Ce(III) is oxidized to Ce(IV), which accompanies Pu(VI) in an S46 process. Thus in diagram C of Table 3, the final step of the upper process will lead to Pu(VI) contaminated with Ce(IV), if an S46 process is chosen instead of the S6 or S34 process. Other similar cases may

be distinguished. This may necessitate more steps than are indicated in Table 3, and is of importance in the selection of a certain unit process.

3. SEPARATION PROCESSES

For the processes given in Table 2, it has generally been assumed that the anions present are non-complexing (*i. e.* ClO_4^-, or perhaps NO_3^-), except for the anions of the reagents. The concentration given for a reagent is the "free concentration", *i. e.* the reagent concentration in excess over the amount complexed by metals present. This is especially important to keep in mind when large amounts of uranium are present.

A large number of methods can be used to precipitate the +3, +4 and +6 oxidation states of the actinides. However, only some familiar methods with a certain degree of specifity are given in Table 2. To obtain complete separation from impurities, several repeated precipitations usually have to be carried out. Dissolving the precipitates constitutes a special problem; the most common methods are digestion with strong acid or alkali (in the latter case followed by an acid treatment), or treatment with a substance that forms a soluble complex with the anion or cation of the precipitate.

In Table 2 only few of many possible extraction processes are given. The decontamination factor from the individual fission products is in most cases better than 100. In some cases (*e. g.* S6b and S46b), however, repeated extractions (3 to 5) must be carried out in order to obtain a yield >99 % in the organic phase, if equal phase volumes are used. In other cases it may be necessary to wash the extract to obtain a good decontamination, *e. g.* in case S46d, where the distribution ratios of some metals are [7]: Pu(IV) 100, Pu(VI) ~3, U(VI) 30, Th 10, Zr, Nb, La and others ≤0.1.

The solubility of the metal complexes formed with organic ligands (S6c, S46c,S4abc) is usually low; the metal complex concentration should therefore possibly not exceed 0.001 M. However, if this concentration is considerably exceeded, so that a precipitate is formed, it may be dissolved by increasing the volume of the organic solvent. In extractions with organic ligands, the distribution ratio of a metal is very sensitive to a change in pH or ligand concentration; the relation between these variables must be understood when such separation processes are used [8].

The solubility and distribution constants vary somewhat for actinides in the same valency state. The precipitation and especially adsorption and liquid-liquid extraction methods can therefore be used for fractionating ions of same valency state. These possibilities have not been indicated in Tables 2 and 3.

Some of the reagents in Table 2 (*e. g.* strong HNO_3, IO_3^-, $H_2C_2O_4$) may effect the redox-potential of the solution, and may make them unsuitable in certain combinations.

The fission products FP[3] (mainly the trivalent rare earths) and FP[4] (mainly Ce(IV) and Zr(IV)) will generally closely accompany the trivalent and tetravalent actinides. The FP[6] fission products include some elements, which follow U(VI). These elements can usually be removed without changing the valency state of uranium, if several repeated separation cycles (*e. g.*, a column extraction) are carried out. After a cooling time of >2 months for the fission pro-

ducts the main part of the FP^x fission products constitutes alkali (Rb, Cs), alkaline earth (Sr, Ba) and noble gas (Kr, Xe) elements. All these elements are removed by most of the separation procedures in Table 2.

Many other processes can be devised for the separation of groups of elements, or to remove troublesome fission products (*e. g.*, sorption processes). A number of such processes have been described in connection with the isolation of actinide elements [e.g.9–13]. The general procedure is only indicated here, and will subsequently be denoted by Sx.

4. GENERAL CONSIDERATIONS ON THE ISOLATION OF PLUTONIUM

When plutonium is isolated on the laboratory scale, the radiation from the fission products may be so low that ordinary chemical operations can be used. The selection between different separation schemes will then only depend on which process is the best from a purely chemical standpoint. In order to obtain a good yield of plutonium, the adsorption of low concentrations of Pu(IV) on glass surfaces must be considered [14,c.f.15]. This adsorption is most pronounced in the region where Pu(IV) hydrolyses, *i. e.* at pH >1. When the Pu(IV) concentration is $>10^{-4}$ M, the adsorption on glass vessels can be neglected. However, in precipitation experiments, the general principles with carriers and hold-back carriers [e.g.16] must be followed, if not considerable amounts of plutonium should be lost.

When plutonium is isolated on an industrial scale, the radiation from large amounts of fission products necessitate chemical operations by remote control [e.g.17]. Some processes, which have been found useful in the laboratory, may then be less practical; for example liquid-liquid extraction processes may be preferred to precipitation processes [18]. Since radiation can decompose chemical compounds [e.g.19], it may be important to remove most of the highly radioactive elements as soon as possible, and also to use compounds, which are less sensitive to radiation decomposition, or which limit the action of the radiation. Several other important questions must also be considered, *e. g.* corrosion problems [20], maintenance of equipment [21], cost of chemicals, *etc.*, which all influence the selection of a separation or redox process. These questions, which mainly are of technological importance, will not in general be considered in the following discussion of the flow-sheets in Table 3.

5. DISCUSSION OF THE COMBINATIONS OF UNIT PROCESSES

Only the simplest combinations of unit processes are given in Table 3. Simpler combinations can in general be devised only at the expense of the purity of some products, *e. g.* the uranium. More complicated combinations involving more steps can of course also be given. However, the complicity of a flow-sheet is not necessarily described by the number of steps in the diagram, where it has been assumed that the separations are practically complete in each process. Since this is generally not true, each of the separation processes has to be repeated several times. A few extra processes may also have to be added.

In most instances, the redox process given can be combined with the previous or subsequent separation process, so as to constitute only one process. For example, in the upper flow-sheet of diagram A in Table 3, the last two processes may be combined so that plutonium is extracted as Pu^3 from an organic solvent containing the $Pu^6U^6FP^6$ mixture into an aqueous solution containing a reducing agent (V36, S^6), thus leaving U^6FP^6 in the organic phase. Such combinations are in general more suitable for liquid-liquid extraction and ion exchange processes than for precipitation processes.

The different combinations of unit processes given in Table 3 will be discussed below in the light of the redox and separation processes presented in Tables 1 and 2.

To obtain Pu(VI) and U(VI) in diagram A, the metal has to be dissolved and treated with a strong oxidizing agent (process V66). Most of the fission products are removed in the first separation step (S6). Of the three following possibilities, the one involving a V34 process is somewhat more complicated, but has the advantage of giving purer uranium. The two other ways are of approximately equal simplicty. In both cases the redox process may be combined with the separation process in the manner described above.

A slight variation of the upper flow-sheet in diagram A of Table 3 may be made, if an S34 process is used instead of the S6 process in the first step. This process will lead to equally pure plutonium [13], but less pure uranium ($U^6FP^{6\times}$).

To obtain Pu(IV) and U(VI) (diagram B, Table 3), the metal is suitably dissolved in HNO_3, or a mixture of HNO_3 and H_2SO_4. By applying an S46 process plutonium and uranium are separated from the bulk of the fission products. Pu^4FP^4 and U^6FP^6 can be separated from each other by an S4 or S6 process. The combination of S46, and S4 or S6 processes can be made in a simple manner; *e. g.* if process S46b is combined with S6b (see Table 2), Pu^4 and U^6 will first be extracted together into the organic (hexone) phase, from which Pu^4 is then washed out when the organic phase is equilibrated with a less acid aqueous phase. The conditions for a process of this kind can of course be chosen much better than happens to be the case in the examples S6b and S46b. The subsequent separation of Pu^4FP^4 can be made in two ways of approximately equal simplicity.

As pointed out previously, a redox and a separation process can be combined into one process. Such a combination in diagram B may be S46, V34, *i. e.* from the mixture of $Pu^4U^6FP^{46}$ obtained in an organic solvent after an S46 process, plutonium is washed out into an aqueous phase with some reducing substance.

The lower flow-sheet in diagram B, which in practice is equivalent to the lower flow-sheet of diagram C, represents the "lanthanum fluoride cycle" [22]. Several repeated cycles of S34, V66, S34, V34 processes have to be carried out to obtain pure plutonium [23].

Processes of the kind shown in diagram A or B seem to be used in practice on a large scale [6,20*].

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* *Added in proof:* At the Geneva Conference on the Peaceful Application of Atomic Energy, August 8—20, 1955, a number of papers describing large scale processes of this type have been declassified (see *e. g. Nucleonics*, September, 1955).

The flow-sheets starting with Pu(III) and U(VI) in diagram C seem to offer no advantage over the flow-sheets previously described. Besides the difficulties, which may be encountered in preparing the Pu^3U^6 solution, all flow-sheets start with a precipitation or sorption step (no S3 or S34 liquid-liquid extraction process has been described), and the bulk of the fission products (FP^3) are not removed until the last separation process.

To obtain Pu(III) and U(IV) in diagram D, the metal has either to be dissolved in a non-oxidizing acid, *e. g.* HCl with some F^- as catalyst (V34d), or to be dissolved in an oxidizing acid and the solution then reduced in an V34 process. The former process constitutes a serious corrosion problem, since stainless steel, and perhaps glass, cannot be used. The latter process involves considerable amounts of reducing agents, since all U(VI) has to be reduced to U(IV). From the solution obtained by either method, $Pu^3U^4FP^{34}$ are removed by a precipitation process. Plutonium is not separated from the bulk of the fission products (FP^3) until the last step. The separation of U^4 from FP^4 can easily be effected by an S4 or S6 process after the oxidation of uranium to U^6.

Of the combinations given in the Table 3, those named A and B seem to be the simplest and most attractive, especially perhaps for large scale work.

The number of chemical methods one might use for each unit process is already considerable, and a great number of other methods could certainly be invented. The number of ways in which a certain scheme such as A or B in Table 3 may be realized is thus great indeed.

However, for each specific combination, one would also have to find out how a number of the minor elements present will behave. Elements with constant oxidation number (like most lanthanides) will cause little trouble, but some transition elements, like Mo and Ru, may change oxidation state in the red-ox processes and then appear at unwanted places. Such elements might need a special separation step. However, considering the very great number of methods possible, it seems probable that combinations could be found that contain no more unit process than do A or B in Table 3.

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Two Methods for the Isolation of Tracer Amounts of Plutonium

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Two new methods have been devised for the isolation of plutonium from uranium samples, which have been irradiated with slow neutrons for about 1 year and have cooled for about 3 months. The number of moles, β- and γ-activity of the products obtained in these samples have been calculated.

The complex formation between organic ligands and some lanthanide and actinide elements has been studied for several years by a group at this institute *e.g.* [1,2]. For a long time it has been our aim to extend these studies to include some transuranium elements. Due to the generosity of the Dutch-Norwegian Joint Establishment for Nuclear Energy Research (J.E.N.E.R.), we have been allowed to irradiate uranium samples in the pile at Kjeller, Norway. Some transuranium isotopes and a number of fission products are formed during this irradiation. This paper describes two methods, which have been found useful for the isolation of plutonium from these samples.

PRODUCTS OBTAINED IN NEUTRON IRRADIATED URANIUM

The radioactivity I_1 (dis./sec.) of an isotope produced by the irradiation of a thin sample by particles of a certain energy can be calculated from the equation

$$I_1 = f\,\sigma\,N\,A_0^{-1}\,a_0\,(1-e^{-\lambda_1 T})\,e^{-\lambda_1 t} = I_0 \cdot e^{-\lambda_1 t} \qquad (1)$$

where f is the particle flux (particles $\times$ cm^{-2} $\times$ sec^{-1}), σ the reaction cross section (cm^2), N the Avogadro number, a_0 the number of grams of the irradiated isotope of the atomic weight A_0 ($N \cdot A_0^{-1} \cdot a_0$ is the number of bombarded atoms), λ_1 the disintegration constant of the product nucleus (half-life $\tau_1 = 0.693\,\lambda_1^{-1}$), T the irradiation time, and t the time between the end of the irradiation and the measurement (cooling time). This equation is valid as long as no macroscopic changes takes place in the target (a_0 = constant), and nuclear reactions with the product isotope can be neglected. For very long half-lives ($\tau \gg T+t$) equation (1) reduces to $I_1 = I_0\,\lambda T$.

If the decay products are also radioactive, their activity can be calculated from the formula first given by Bateman [3]

$$I_n = I_0 \ (C_1 \, e^{-\lambda_1 t} + C_2 \, e^{-\lambda_2 t} + \ldots\ldots + C_n \, e^{-\lambda_n t}) \qquad (2)$$

where

$$C_1 = \frac{\lambda_1 \cdot \lambda_2 \cdots \lambda_{n-1}}{(\lambda_2 - \lambda_1)(\lambda_3 - \lambda_1) \cdots (\lambda_n - \lambda_1)}$$

$$C_2 = \frac{\lambda_1 \cdot \lambda_2 \cdots \lambda_{n-1}}{(\lambda_1 - \lambda_2)(\lambda_3 - \lambda_2) \cdots (\lambda_n - \lambda_2)}$$

$$C_3 = \frac{\lambda_1 \cdot \lambda_2 \cdots \lambda_{n-1}}{(\lambda_1 - \lambda_3)(\lambda_2 - \lambda_3) \cdots (\lambda_n - \lambda_3)}$$

etc. which is valid for $n > 1$.

Tables and methods to simplify the solvation of these equations have recently been given by Flanagan and Senftle [4], and by Kirby and Kremer [5].

The mass a_i in grams of a particular isotope produced is given by

$$a_i = I_i A_i / \lambda_i N \qquad (3)$$

In combining eqn (1) with eqn (3) it should be noted that A_0 i eqn. (1) is the mass number of the bombarded nucleus, while A_i in eqn. (3) is the mass number of the product nucleus. In most nuclear reaction $A_i \approx A_0$. All time should be given in seconds in eqns. (1)—(3).

Equations (1)—(3) have been used to calculate the composition of the products formed in 20 g UO_3 samples, which have been irradiated with 9×10^{18} thermal neutrons per cm^2 ($f \cdot T$) for about a year (T), and have cooled for about 3 months (t). The results are given in Table 1.

Table 1. Main products in 20 g UO_3, which has been subjected to slow neutron irradiation; $f = 3 \cdot 10^{11}$ neutrons $\times$ cm^{-2} $\times$ sec^{-1}, $T = 1$ year, $t = 3$ months.

Isotopes	Amount	Radioactivity
$U^{238} + U^{234}$	16.6 g	$2.5 \ 10^7$ α-dpm
Np^{237}	$<10^{-6}$ g	600 α-dpm
Pu^{239}	$0.42 \ 10^{-3}$ g	$6.0 \ 10^7$ α-dpm
Th^{234} (UX_1)	$<10^{-6}$ g	$1.2 \ 10^7$ β-dpm
Pa^{235} (UX_2)	$<10^{-6}$ g	$1.2 \ 10^7$ β-dpm
Fission products	$0.57 \ 10^{-3}$ g	$5 \ 10^{11}$ β-dpm *
		$3 \ 10^{11}$ γ-particles/min **

* 0.23 curie.

** γ-energy $\geq$0.1 MeV.

The relative amounts and fractions of β- and γ-activity of each fission product has also been calculated, and the results are given in Table 2. Since the fine structure for only one of the peaks [6] of the fission yield curve was available, the calculations have been based on the smooth fission yield curve [7].

Table 2. The percentage of moles, β- and γ-activity of fission products obtained after an irradiation time of 1 year and a cooling time of 3 months.

											Zn	Ga	Ge	As	Se	Br	Kr
											—	—	0.001	0.001	0.10	0.077	0.25
											—	—	—	—	—	—	0.38
											—	—	—	—	—	—	—
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	J	Xe
1.51	5.8	2.35	15.6	0.28	11.6	2.83	6.00	1.73	0.76	0.015	0.033	0.005	0.040	0.027	1.17	0.61	8.0
0.46	8.7	10.9	13.3	21.9	—	—	5.6	1.19	—	—	0.007	—	0.023	0.035	0.49	0.005	—
0.23	—	—	22.1	37.0	—	—	7.0	0.70	—	—	0.011	—	0.001	0.15	1.40	0.008	0.004
Cs	Ba	La										Tl	Pb	Bi	Po	At	Rn
8.4	4.8	(see															
0.86	0.27	be-															
0.61	1.26	low)															
Fr	Ra	Ac	Th	Pa	U	Np	Pu			0.015	0.033	0.005	0.041	0.028	1.27	0.69	9.3
										—	0.007	—	0.023	0.035	0.49	0.005	0.38
										—	0.011	—	0.001	0.15	1.40	0.008	0.004
9.9	10.6	29.4	15.6	0.28	11.6	2.83	8.5										
1.32	9.0	46.7	13.3	21.9	—	—	6.8										
0.84	1.26	29.5	22.1	37.0	—	—	7.7										

La	Ce	Pr	Nd	Pm	Sm	Eu	Gd	
3.29	7.8	3.15	10.0	1.37	1.29	0.09	0.012	27.0
0.27	18.4	14.4	0.08	2.60	0.002	0.06	—	35.8
0.76	5.1	23.6	0.04	—	—	0.001	—	29.5

KEY	
Zr	element
15.6	mole %
13.3	% β-activity
22.1	% γ-activity (E > 0.1 MeV)
15.6	sum of mole % in Sub-group
13.3	» » % β-activity » »
22.1	» » % γ-activity » »

The true values can be obtained by multiplying the figures in the table by the ratio between the measured and smooth yield-values. This ratio usually lies between 0.7 and 1.3 [6]. Only γ-energies above approximately 0.1 MeV have been included.

The values for the fission products in Table 1 are obtained from the sum of the individual fission products. This sum agrees well with values which can be calculated for the unseparated fission products [8,9]. From a knowledge of the γ-ray spectra for the unseparated fission products (~ 30 % 0.1—0.3 MeV, ~70 % 0.5—0.8 MeV), one can calculate [10] that the radiation intensity obtained at a distance of 1 meter from the 0.57 mg of fission products in the 20 g UO_3 samples is 40 milliroentgens per hour.

The nuclear constants, which have been used for the calculations in this section, have been obtained from recent compilations [11-15].

EXPERIMENTAL

Depending on the activity of the samples, the chemical experiments were carried out either in an iron-lead shielded box [16], in a dry-box or in well-ventilated fume hoods.

All chemicals used were of p.a. quality. UO_3 of high purity was obtained from A. B. Atomenergi; it decomposed partly during the pile irradiation.

The α-samples were prepared by the evaporation of ≤ 0.10 ml of solution on Pt-dishes, placed on aluminium rings on a hot plate. In order to obtain thin and smoothly spread layers of the samples in the presence of visible amounts of salts, the samples were slurried with glycerol and slowly dried at 250° C. The solid matter of the samples never exceeded 0.3 mg/cm^2, so that the self-absorption of α-particles in the samples could be neglected [27].

All measurements were made with a gas-flow (90 % A, 10 % CH_4) proportional counter (Nuclear Measurements Corp., Indianapolis); the samples and the Al-foils used in the absorption experiments were measured inside its hemi-spherical ionization chamber (radius 3 cm).

THE ISOLATION OF PLUTONIUM

The object of the isolation may either be to obtain chemically pure or radioactively pure plutonium. For tracer chemical work with plutonium, the latter object is usually more important; it will generally also provide a sufficient chemical purity of the product.

The most common laboratory method used in the isolation of plutonium from neutron irradiated uranium is the "lanthanum fluoride cycle" [17]. However, a great number of cycles (five to ten [18]) have to be carried out to obtain radioactively pure plutonium. The use of highly corrosive HF is a further drawback of the method.

Many other methods have been, and can be devised, which involve fewer steps and no HF. The two methods, which are described below, give radioactively pure plutonium in relatively few steps. These methods do not appear to have been described previously.

Separation method I

The "zirconium phosphate cycle" devised for the isolation of neptunium [19] has been modified to suit the isolation of plutonium. Instead of zirconium, bismuth is used as a carrier for the phosphate precipitates. Some of the advantages of this change are that the bismuth phosphate is easier to filter and to dissolve than the zirconium phosphate. Depending on the conditions in the solution, the bismuth phosphate can be made to carry either the +3 and +4 valency states of the actinides together, or only the +4 valency state.

As a final purification step, an extraction with the chelating agent thenoyltrifluoroacetone is used.

Extraction processes with chelating agents have the advantage that very high decontamination factors can be achieved. Thus it can be shown [20] that the distribution ratio of Th(IV) between 0.3 M thenoyltrifluoroacetone in benzene and 0.2 M HNO_3 is about 10^{12} times greater than the distribution of La (III) between the solvents. Though it is usually not possible to separate the phases with so great a precision that the quotient between the distribution ratios will become equal to the decontamination factor, a factor $> 10^4$ is easily reached.

With the symbols used in the preceding paper [21] the separation flow-sheet consist of the following unit processes: V46, V66, S34, V36, S34, V46, S4.

1. The uranium sample is dissolved in hot, conc. HNO_3 and kept at 90° C for 30 minutes, giving a solution of U(VI) and Pu(IV) [22]. (V46.)
2. The solution is diluted to 5 M in HNO_3 and solid $NaBiO_3$ is added to oxidize plutonium to the +6 valency state. (V66.)
3. The solution is made 0.1 M in HNO_3 (if it becomes turbid, HNO_3 is added until the solution is clear) and 0.1 M in phosphate. The $BiPO_4$ precipitate is centrifuged off. This precipitate carries most of the fission products, and especially those with a chemistry similar to Pu(IV). (S34.)
4. The supernatant solution containing Pu(VI) is made 1 M in HNO_3, 0.05 M in N_2H_4 and 0.005 M in Fe^{3+}. In this solution plutonium is rapidly reduced to Pu(III). (V36.)
5. The solution is diluted to 0.1 M in HNO_3 and made 0.1 M in phosphate. Bi^{3+} is then added (1 drop 0.1 M Bi^{3+} for each ml solution) to precipitate $BiPO_4$, which carries all the plutonium. The precipitate, which is usually contaminated with some uranium, is centrifuged off and washed. (S34.)
6. The phosphates in the precipitate are converted into hydroxides by treating with warm ~10 M KOH. After centrifuging, the solution is drawn off, and the precipitate washed and dissolved in hot, conc. HNO_3. (V46.)
7. After some hours at room temperature, the solution is diluted to 1 M HNO_3, and extracted with an equal volume of 0.3 M thenoyltrifluoroacetone in benzene. This gives a pure Pu(IV) solution in the benzene phase, leaving the rest of the uranium and the fission products in the aqueous phase (see also steps 5 and 6 in separation method II, below). (S4.)

The plutonium yield of the preliminary experiments with this method in our laboratory has been better than 60 %. No β-γ-activity above background could be detected in the final plutonium sample, when measured through a 10 mg/cm^2 Al-absorber.

Separation method II

From Table 2 of the fission products it is seen that there is a great number of elements to the right of Nb, which can appear in many different oxidation states, so that it may be difficult to predict their behavior in a certain separation scheme. However, most of these elements have sulfides, which are insoluble in fairly strong acids [23]. The other elements to the right of Nb either form anions or gaseous products when dissolved in strong HNO_3, and can thus be separated from the cations to the left of Nb by relatively simple methods. It was therefore felt that many troublesome fission products could be removed with a sulfide precipitate in HNO_3, provided that a suitable carrier is used.

It is seen from Table 2 that Zr and Nb constitute about 35 % of the total β-activity and about 60 % of the total γ-activity of the fission products. Since Zr in many respects behaves chemically like Pu(IV), the separation of Zr from Pu is one of the most important steps in the purification of plutonium.

In experiments with tracer Zr and Nb it was found that a considerable amount of these elements was adsorbed on glass surfaces. This adsorption depends upon the acid used, and varies with the concentration of the acid. Fig. 1 shows the adsorption of a mixture of Zr^{95} and Nb^{95} on a silica gel column as a function of acid concentration. No attempt to separate Zr and Nb were made in these experiments. Since plutonium does not adsorb in such a column at HNO_3 concentrations above 2 M, a SiO_2-adsorption step was incorporated in the separation procedure.

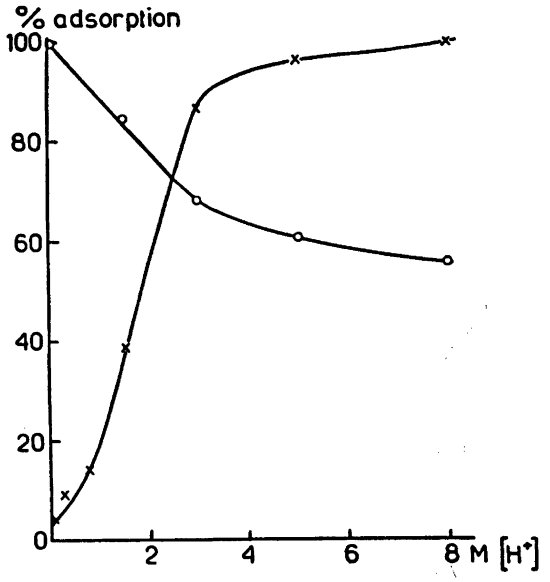

Fig. 1. The adsorption of tracer Zr+Nb on a silica gel column 10 cm long and 0.8 cm wide as a function of HCl (O) and HNO_3 (×) concentration.

As a final purification step, the thenoyltrifluoroacetone extraction process was used.

With the symbols used in the previous paper [21], the flow-sheet consists of the following processes: V46, Sx, V46, S4.

1. The UO_3 sample is dissolved in hot, conc. HNO_3 and kept at 90° C for 30 minutes, giving a solution of U(VI) and Pu(IV) [22]. (V46.)
2. The acidity of the cooled solution is adjusted to 6 M in HNO_3, and the solution is run through two columns of SiO_2 gel. This removes most of the Zr and Nb. The columns have a diameter of 0.8 cm and a length of 10 cm. (Sx.)
3. Cu^{2+}, La^{3+} and ZrO^{2+} (1 drop 0.1 M carrier for each ml of solution) are added to the solution, and it is then diluted to 0.5 M in HNO_3. On the addition of H_2S, CuS precipitates and carries most of the fission products with sulfides insoluble in 0.5 M HNO_3. The precipitate is centrifuged off. (Sx.)
4. The solution is evaporated almost to dryness and then kept for 30 minutes in hot ~10 M HNO_3. This removes H_2S and restores the Pu(IV) valency state. (V46).
5. The solution is diluted to 1 M in HNO_3 and extracted with an equal volume of 0.3 M thenoyltrifluoroacetone in benzene. During these conditions practically only Pu(IV) and Zr(IV) are extracted, leaving uranium, thorium (UX_1) and the rest of the fission products in the aqueous phase [20, 24]. (S4.)
6. After a two-fold dilution of the organic phase with benzene, Pu(IV) can be extracted back into an aqueous phase with 10 M HNO_3 or $HClO_4$. This provides an additional purification from Zr [20]. In practice it has not proved necessary to use this step.

The plutonium yield of this procedure was about 90 % in the preliminary experiments performed in this laboratory. The decontamination from β-γ-radiation of the fission products is approximately 10^6 (see also below).

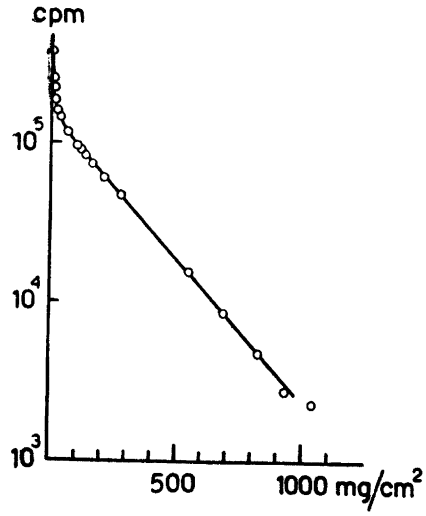

Fig. 2. Measured radioactivity (cpm) from neutron irradiated uranium sample as a function of aluminium absorber thickness (mg/cm²).

ABSORPTION MEASUREMENTS

The purity of the samples was checked by measuring the absorption of the radiation through aluminium foils.

The absorption curve obtained for the original, neutron irradiated UO_3 sample is shown in Fig. 2, and Fig. 3 gives the absorption curve for the isolated plutonium sample (*i. e.* the benzene phase of step 5 in separation method II, above) *. The activity of the Pu-sample measured without an adsorber was 7 600 $\pm$ 80 cpm (counts per minute). Both curves are taken on the β-plateau of the proportional counter. The α-activity of the Pu-sample (measured on the α-plateau of the proportional counter) was 1 220 $\pm$ 10 cpm. The difference between these two activities, 6 380 $\pm$ 90 cpm, is most probably due to soft β-radiation. The range of this radiation is about 7 mg/cm ^{2}, corresponding [25] to a maximum β-energy of about 70 keV. The range also corresponds [26] to an α-energy roughly equal to that of Pu ^{239}, but the ratio between the activities measured on the β-plateau and the α-plateau excludes the possiblity that the absorption curve in Fig. 3 is only due to the α-particles. In addition the sample contains some γ-radiation. The ratio between the α-, β- and γ-activities measured in the ionization chamber is about 1:5:0.01. From Table 1 and Figs. 2 and 3 it can be found that the final plutonium sample contains less than 1:10 ^{6} of the fission products with a β-energy >0.07 MeV.

The author wishes to thank the head of FOA 1, Prof. G. Ljunggren, for his kind interest in this work, and Mrs. B. Olausson for experimental aid.

* The ordinate values of the curves are not comparable.

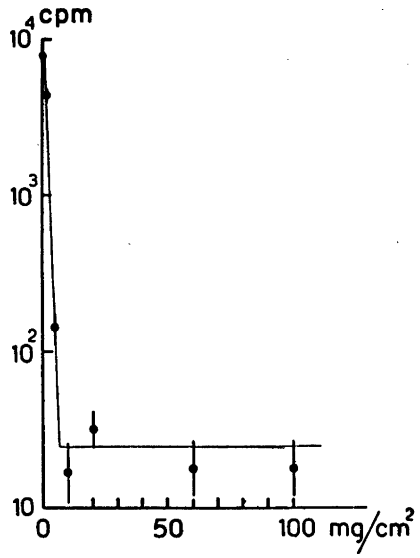

Fig. 3. Measured radioactivity (cpm) from the isolated plutonium sample as a function of aluminium absorber thickness (mg/cm²).

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Deuterium Isotope Effect on the Aquation and Hydrolysis Rates of Aqueous $[Co(NH_3)_5Cl]^{+2}$ and $[Co(NH_3)_5Br]^{+2}$

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The hydrolysis of $[Co(NH_3)_5Cl]^{+2}$ and the aquation of this and of $[Co(NH_3)_5Br]^{+2}$ have been studied in both ordinary and in deuterium containing aqueous systems. The hydrolysis rate in heavy water was some sixty percent of that in ordinary water, an effect due primarily to a lower frequency factor. The aquation rates were similarly lower in deuterium containing systems, and, because of the slow exchange between complex and solvent in acid media, it was possible to observe the four possible aquation rates corresponding to light or heavy complex in light or heavy solvent.

From a consideration of the deuterium effect and also the large entropy of activation, 37 E.U., as well as of other aspects, it is concluded that the hydrolysis proceeds through an acid base pre-equilibrium. For the aquation reaction, an explanation of the deuterium effects is given, involving a "front side" replacement by water, facilitated by hydrogen bond bridges.

The aquation and hydrolysis *** of complex ions have been rather extensively in vestigated, but their mechanisms are still a subject for active speculation. The reader is referred to a recent review paper [1] for a summary of the problems involved and a more complete coverage of the literature.

The present investigation relates to the type reactions:

$$[CoA_5X]^{+2} + H_2O = [CoA_5(H_2O)]^{+3} + X^- \quad (1)$$

$$[CoA_5X]^{+2} + OH^- = [CoA_5(OH)]^{+2} + X^- \quad (2)$$

The aquation reaction (1) follows first order kinetics [2-4], but the mechanism has been considered by some to be S_N1 (rate determining dissociation to a penta-

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*** Some brief can be made for terming these reactions acid and base hydrolysis, but the older usage will be adhered to here.

coordinated intermediate) [5-7], and by others, to be S_N2 (bimolecular substitution)[8]. Similarly, although the hydrolysis reaction (2) is second order [9,10], the mechanism has been supposed to be S_N2 by some investigators [8], and, by others, to involve an acid-base pre-equilibrium [11,12,13].

Substitution of deuterium for hydrogen in reacting systems introduces various types of effects in a way that frequently makes it possible to distinguish between kinetically similar mechanisms [13-15]. The present investigation of the deuterium effect on the rates of aquation and hydrolysis of $[Co(NH_3)_5Cl]^{+2}$ and $[Co(NH_3)_5Br]^{+2}$ was undertaken with this in mind.

EXPERIMENTAL PROCEDURES

Preparation of compounds. The cobalt(III) complexes were kindly supplied by Mr. C. E. Schäffer, who had prepared them by the procedures of S. M. Jørgensen [16]. The compounds were recrystallized, washed with water, alcohol, and ether, and air dried; the $[Co(NH_3)_5(H_2O)]Cl_3$ was kept under refrigeration.

The preparation of $[Co(ND_3)_5Cl]Cl_2$ was as follows. A solution of $[Co(NH_3)_5(H_2O)]Cl_3$ in 99 % D_2O was made alkaline by the addition of metallic sodium, and the resulting solution of $[Co(ND_3)_5(OD)]^{+2}$ was allowed to stand for several hours. It was then acidified with dry hydrogen chloride gas, and digested on a steam bath until the precipitation of $[Co(ND_3)_5Cl]Cl_2$ was fairly complete. The product was then washed and air dried. Information about its deuterium content was obtained from a comparison of its infra red spectrum with that for the ordinary complex, using samples of powdered material in *per*-chlorobutadiene. The ordinary and the deuterated complex showed strong bands at 3.00 and 4.14 microns, respectively, as expected for the N—H and the N—D bond [17]. The infra red measurements were made by Mrs. S. Refn through the courtesy of Professor S. Veibel. Some of the D_2O was kindly made available by Professor R. W. Asmussen.

While the above results were quite qualitative, exchange data, to be described below, indicated a deuterium content of 90 % for the heavy complex.

Exchange studies. Qualitative data on the rates of exchange of deuterium between $[Co(ND_3)_5Cl]^{+2}$ and H_2O under acid and alkaline conditions, were obtained as follows. For the former, a 0.02 M solution of the complex at pH 3 and at 38° C, was employed. The deuterium content of the solvent was found to rise slowly with time, and corresponded to about 18 % exchange after two hours. For the second experiment, a similar solution of complex was employed, but 0.01 M in sodium hydroxide and kept at 18° C. Here exchange appeared to be complete in 1.5 minutes, and corresponded to a deuterium content for the complex of 90 %. Anderson *et al.*[11] observed a similar exchange behavior for $[Co(NH_3)_6]^{+3}$.

The actual deuterium analyses were done by the gradient tube technique [18], and were carried out at the Carlsberg Laboratory, through the courtesy of Professor Linderstrøm-Lang.

Aquation runs. For the aquation rate studies, a solution of the light or heavy complex in light or heavy water ("heavy" denoting about 85 or 90 % deuteration) was prepared, and made 0.1 M in nitric acid. A 1 cm absorption cell was filled with the solution, which had been pre-heated to the desired temperature, and the covered cell was then placed in the thermostatted cell holder of a model DU Beckman spectrophotometer. The temperature control, as determined by direct measurements on the solutions in the cells, was good to $\pm 0.1°$. Since two or three runs were generally made simultaneously, comparisons between them were not very dependent upon the quality of temperature control.

The course of the reaction was followed by measuring the time variation of the optical density at 550 mμ, and the data were plotted according to the first order rate law,

$$\log [(D-D\infty)/(D^\circ-D\infty)] = -\frac{kt}{2.303} \qquad (3)$$

where D denotes optical density. The appropriate value for $D\infty$ was determined from separate extinction coefficient determinations, described below.

Hydrolysis runs. The hydrolysis runs were generally carried out as follows. A solution of the chloropentammine complex of the desired concentration was prepared, and its optical density checked. This solution and one of 0.1 M sodium hydroxide were then brought to the desired temperature and equivalent quantities of the two were mixed and immediately placed in a cell waiting in the thermostatted cell block. Due regard was given to the heat of mixing effect; other procedural details were similar to those for the aquation runs.

The rate was measured by following the optical density at 500 mμ, the data being plotted according to the second order rate law,

$$(D-D^\circ) / (D\infty - D) = C^\circ kt \qquad (4)$$

where C° denotes the equal concentration of complex and of base.

The procedure for the runs with excess base was quite similar, except that 1 M rather than 0.1 M alkali was employed, and the data were plotted according to (3), the slope of the plot now giving the product of the second order rate constant and the constant concentration of base.

For the hydrolysis of $[Co(ND_3)_5Cl]^{+2}$ in D_2O, it was only necessary to dissolve ordinary complex in heavy water, since exchange was rapid once the solution was made alkaline.

It might be noted that it appeared desirable to use very pure sodium hydroxide since otherwise irreproducible and generally high rates resulted.

Determination of extinction coefficients. In general, it was not possible to determine $D\infty$ values from the aquation and hydrolysis rate studies themselves. Some decomposition, with loss of ammonia, would set in before the reactions reached the necessary degree of completion, giving incorrect empirical values of $D\infty$. The only exception was that of hydrolysis in excess base, which was both rapid and first order.

The difficulty was circumvented by employing $D\infty$ values calculated from the extinction coefficient separately determined for the assumed product, *i. e.* $[Co(NH_3)_5(H_2O)]^{+3}$ or $[Co(NH_3)_5(OH)]^{+2}$. It was found that the extinction coefficients themselves were subject to an isotope effect, and the complete set of ε values is given in Table 1.

The complete absorption spectra for complexes in deuterated systems were actually only slightly different, as determined on a Cary recording spectrophotometer. For example, the 550 mμ peak for $[Co(NH_3)_5Cl]^{+2}$ was shifted about 5 mμ toward the blue in the all deuterium system. However, at the wave lengths of 500 and 550 mμ employed for the rate measurements, significant differences did materialize in some cases, using the DU Beckman spectrophotometer. As might be expected, the isotope shifts were greatest for wave lengths midway between an absorption maximum and minimum, as in the case of the aquopentammine at 550 mμ, and least when the wave length corresponded to a maximum. (As an aid in orientation, the absorption maxima for $[Co(NH_3)_5Cl]^{+2}$, $[Co(NH_3)_5(H_2O)]^{+3}$, and $[Co(NH_3)_5(OH)]^{+2}$ occur at 535, 492, and 500 mμ, respectively.) In general the presence of deuterium either in solvent or in complex shifted the absorption spectrum towards shorter wave lengths.

The values of ε in Table 1 were obtained as follows. For the chloro complex, the desired amount of the appropriate form was weighed out, dissolved in 0.1 M nitric acid, and the extinction coefficient calculated from the measured optical density.

Two series of ε values for the aquopentammine and hydroxypentammine complexes were obtained by starting with a 0.2 M solution of $[Co(NH_3)_5(H_2O)]Cl_3$. This was diluted to 0.01 M either with ordinary or with heavy water, and the optical density measured. The solution was then made alkaline with a small excess of concentrated sodium hydroxide solution, giving either $[Co(NH_3)_5OH]^{+2}$ or $[Co(ND_3)_5(OD)]^{+2}$, exchange with solvent being rapid under these conditions, and the density again determined, allowance for dilution being made in computing the ε values. Finally, the solution was acidified with a small excess of concentrated nitric acid, giving $[Co(NH_3)_5(H_2O)]^{+3}$ or $[Co(ND_3)_5(D_2O)]^{+3}$, and again measured.

Two similar series of measurements were also carried out with a 0.2 M solution of the aquocomplex in heavy water, which had been made alkaline and then reacidified, so that deutero complex was present.

Table 1. Isotope effect on extinction coefficients.

Complex [a,b]	*Substrate* [a,c]	*Extinction coefficients* [d]	
		550 mμ	*500 mμ*
$[Co(NH_3)_5Cl]Cl$	H_2O	46.3, 47.2 *46.8*	42.2, 42.0 *42.1*
	D_2O	*46.5*	
$[Co(ND_3)_5Cl]Cl_2$	H_2O	44.6, 44.8, 46.2 *46.5*	42.0, 40.2, 41.1 41.1 *41.1*
	D_2O [e]	*47.6*	*43.8*
$[Co(NH_3)_5H_2O]Cl_3$	H_2O	21.1, 20.9 *21.0*	46.9, 47.1 *47.0*
	D_2O	*18.5*	*44.9*
$[Co(ND_3)H_2O]Cl_3$	H_2O	*19.2*	*44.8*
$[Co(ND_3)D_2O]Cl_3$	D_2O	19.1, 19.0, 19.4 *19.2*	45.3, 45.1, 45.7 *45.3*
$[Co(NH_3)_5OH]Cl_2$	H_2O		66.7, 66.9 *66.8*
$[Co(ND_3)_5OD]Cl_2$	D_2O		67.1, 69.2, 69.2 *69.2*

a) Where deuterium is indicated, the percent deuteration was *ca.* 85 %.
b) Only two attainable systems exist for the hydroxypentammine complex because of the rapid exchange with solvent. The complex was *ca.* 0.01 M in all cases.
c) Values for the chloro and aquo complex were measured in *ca.* 0.1 M nitric or hydrochloric acid, while those for the hydroxy complex were for solutions *ca.* 0.01 M in sodium hydroxide.
d) Individual values represent completely independent determinations.
e) These values are based on run 55 where a reliable determination of $D\infty$ was possible.

EXPERIMENTAL RESULTS

Comparison of hydrolysis rates as determined spectrophotometrically and titratometrically. The spectrophotometric method which was employed for the majority of the rate studies required the use of a calculated $D\infty$ value, as discussed in the preceding section. It was therefor considered desirable to verify that the assumed reaction,

$$[Co(NH_3)_5Cl]^{+2} + OH^- = [Co(NH_3)_5OH]^{+2} + Cl^- \qquad (5)$$

was indeed occurring to the practical exclusion of appreciable amounts of other processes and that the spectrophotometric method was not subject to some systematic error.

Two additional methods were therefor employed as a check. The first of these comprised a determination of the rate of consumption of hydroxide ion with time, carried out by first adding excess acid to the sample, to quench the reaction, and then back titrating with base to a thymol blue end point. At the pH of this end point, the aquopentammine complex is not titrated [19]. As shown in Fig. 1, substantial agreement was found between this and the spectrophotometric method. The second check involved a titration for chloride ion, using mercuric nitrate solution as reagent, and nitroprusside ion as indicator, and, again, as shown in Figure 2, satisfactory agreement was obtained.

Determination of the order of the hydrolysis reaction. Having established that equation (5) did in fact represent the course of the reaction over its major portion, it was of interest to verify the kinetics. While there is existing evidence

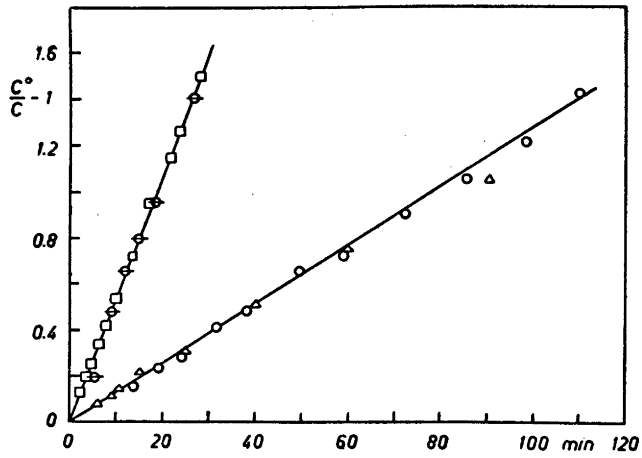

Fig. 1. Hydrolysis of $[Co(NH_3)_5Cl]Cl_2$ O*-Run 40 (spectrophotometric, at 3.3°);* △ *-Run 38 (chloride titration, at 4.0°);* □ *-Run 42 (spectrophotometric, 11°);* ⊖ *-Run 29 (hydroxide titration, 11°).*

that the reaction should be second order, some deviations from this kinetics might develop at higher base concentrations if the mechanism were not of the simple S_N2 type.

The collected results are illustrated in Figs. 2 and 3, and summarized in Table 2. Two series of runs were made. For the first, the starting solutions were made up to be of equal concentration, $C°$, in complex and in base, and it

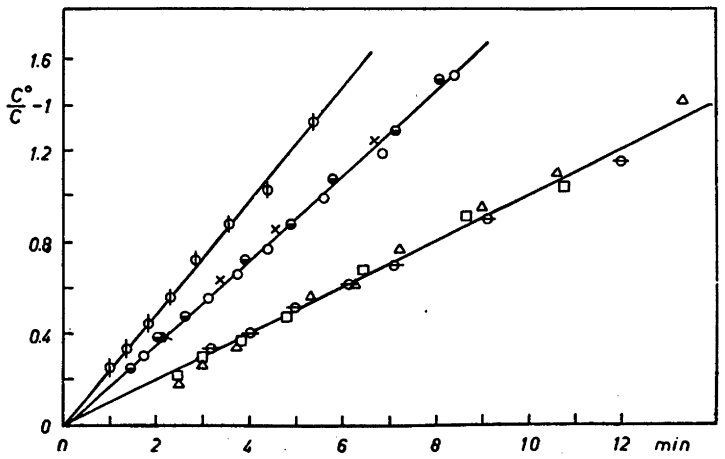

Fig. 2. Hydrolysis of $[Co(NH_3)_5Cl]Cl_2$ *at 18° C.* □, ⊖, △ *-Runs 7, 8, 14, C = 0.00650;* × *-Run 37 (chloride titration, C = 0.01183)* 0 O, ◒*-Runs 43, 46, C = 0.01183;* Φ*-Run 13, C = 0.0140 (19° C).*

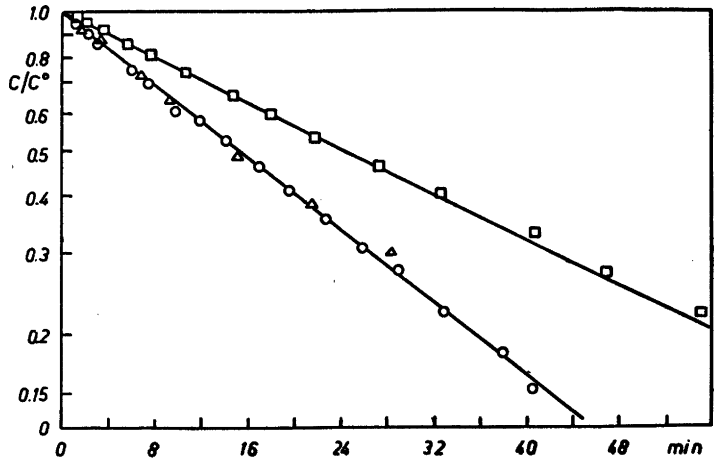

Fig. 3. Hydrolysis of $[Co(NH_3)_5Cl]Cl_2$ at 2.5° C in excess base. O, △*-Runs 53, 54;* □ *-Run 55 ($[Co(ND_3)_5Cl]Cl_2$ in D_2O. $C_{complex}$ = 0.0050 M, C_{NaOH} = 0.0502 M.*

is seen that the data obeyed equation (4) and that essentially constant values of $k_{2\text{-order}}$ were obtained at 18° over a two fold variation in C°. For the second set, a twenty fold excess of base was employed, and, to keep the velocity

Table 2. Hydrolysis rates for $[Co(NH_3)_5Cl]Cl_2$ and $[Co(ND_3)_5Cl]Cl_2$.

Run Nos.	t° C	Concentrations (M)		Rate Law (Equation No.)	Slope of Rate Plot (min^{-1})	$k_{2_1\text{-order}}$ (M^{-1} min^{-1})
		Complex	NaOH or NaOD			
$[Co(NH_3)_5Cl]Cl_2$ in H_2O						
47	25.3	0.0118 $_3$	0.0118 $_3$	4	0.61	51.6
7, 8, 14	18.0	0.00650	0.00650	4	0.100	15.4
37, 43, 46	18.0	0.0118 $_3$	0.0118 $_3$	4	0.18	15.2
13	19	0.0140	0.0140	4	0.244	17.4 (15) [a]
						Av. 15.2
29, 42	11.0	0.0118 $_3$	0.0118 $_3$	4	0.053	4.48
38, 40	3.6	0.0118 $_3$	0.0118 $_3$	4	0.0129	1.09
53, 54	2.5	0.0050	0.0502	3	0.0192	0.91 (1.1) [b]
$[Co(ND_3)_5Cl]Cl_2$ in D_2O						
44	18.0	0.0114	0.0118 $_8$	6	0.00192	9.2
48	17.6	0.0114	0.0118 $_8$	6	0.00155	7.4 (8.0) [c]
						Av. 8.6
55	2.5	0.0050	0.0502	3	0.0124	0.57

a) corrected to 18° C. b) corrected to 3.6° C. c) corrected to 18° C.

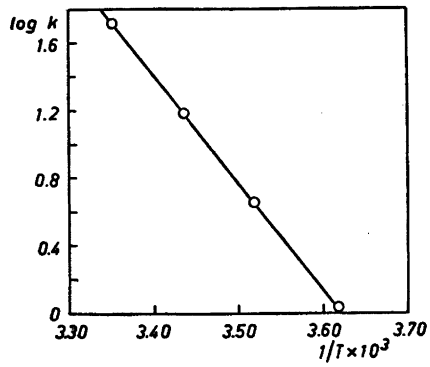

Fig. 4. Temperature dependence of the hydrolysis rate constant for $[Co(NH_3)_5Cl]Cl_2$.

manageable, a temperature of 2.5°. As seen from Table 2, the second order rate constant computed from the application of equation (3) to the data ($k_{2\text{-order}}$ = (slope)/(base)) agreed well with that obtained from data for one fourth the concentration of base and obeying the second order rate law (4).

Between the two series, agreement with second order kinetics is indicated for the range of hydroxide ion concentration from 0.0065 to 0.05 M.

Temperature dependence of the hydrolysis reaction. Temperature dependence data have not previously been reported for the hydrolysis of $[Co(NH_3)_5Cl]^{+2}$, and for this reason the present reaction was studied at several temperatures. The data are included in Table 2, and the plot of log k *versus* $1/T$ is shown in Fig. 4. The slope corresponds to an activation energy of 28.7 Kcal/mole. This is quite high for so fast a reaction, and the entropy of activation, correspondingly is also high. At 18°, using the molar standard state, it is 37 cal/° mole.

Hydrolysis rate of $[Co(ND_3)_5Cl]^{+2}$ *in* D_2O. Two hydrolysis runs were made with the deuterated system, under conditions such that the concentrations of complex and of base should have been equal. The rate law (4) was not obeyed, however, and the difficulty was traced to the assumption that the extinction coefficient would be the same as in the non-deuterated system; this led to the examination of isotope effects on ε values, reported in the preceding section. When the correct values were used, recalculation of the data necessitated the use of the general second order rate equation,

$$\log \frac{b}{a}\left[\frac{C}{(b-a)+C}\right] = X = \frac{(a-b)\,kt}{2.303} \qquad (6)$$

where a and b denote the initial concentrations of complex and of base, and C, that of the complex at time t. The data were now found to give linear plots of X *versus* t and the rate constants so calculated are included in Table 2.

In addition, the rate in heavy water was determined under the conditions for first order kinetics, *i. e.* with excess base, at 2.5°, and these results are shown in Fig. 3, and given in Table 2.

Table 3. Rates of aquation of $[Co(NH_3)_5Cl]^{++}$ and of $[Co(ND_3)_5Cl]^{++}$ in ordinary and in heavy water.

Run Nos.	Isotopic Content [a]		Concn. of Complex	$t°$ C	Half Life (min)
	Complex	Solvent			
20	H	H	0.0201	52	265
52	H	H	0.0137	52	265
56	H	H	0.0119	53.2	253 (262) [b]
					264
50	D	H	0.0133	52	385
57	D	H	0.0123	52.3	365 (378) [c]
					382
21	H	D	0.0195	52	422
51	D	D	0.0140	52	365

a) Where deuterium is indicated, the percent deuteration was *ca.* 85 to 90.
b) corrected to 52°. c) corrected to 52°.
Note: Runs 20 and 21, Runs 50, 51 and 52, and Runs 56 and 57 were carried out simultaneously in adjacent cells in the spectrophotometer.

The hydrolysis rate in heavy was 56 and 63 % of that in ordinary water, at 18° and 2.5°, respectively. The experimental error was such however, that the difference between these two figures is not an accurate indication of the change in activation energy on deuteration. However, the direction of the variation corresponds to a lesser activation energy for the deuterated system, so that in order to account for the smaller rate, the entropy of activation must also be less positive.

Rates of aquation of $[Co(NH_3)_5Cl]Cl_2$ and of $[Co(ND_3)_5Cl]Cl_2$ in ordinary and in heavy water. Aquation rates were obtained at 52° C and pH = 1, by the spectrophotometric method, for systems constituted in four different ways. These comprised light or heavy complex dissolved in light or heavy water. The results are presented in Fig. 5 and summarized in Table 3.

The data which were obtained on the rate of exchange of deuterium between $[Co(ND_3)_5Cl]^{+2}$ and water indicated it to be slow but measurable at 38° and pH = 3 (see Experimental procedures). Although the present runs were made at 52°, the pH was much lower, so some uncertainty existed as to the exchange rate. However, a comparison of runs 20, 52, and 56 with runs 50 and 57 clearly showed that the rates of aquation of the normal and the deuterated complex in ordinary water were different. This would not have been the case had there been exchange. Moreover, the first order plot was obeyed up to more than one half life, so that only a partial exchange could have taken place during this period. It was necessary to establish this point, since the choice of ε values depended upon whether or not exchange was assumed to have occurred. It should be added that runs 50 and 57 were slower than their counterpart in ordinary water regardless of which set of ε values was employed.

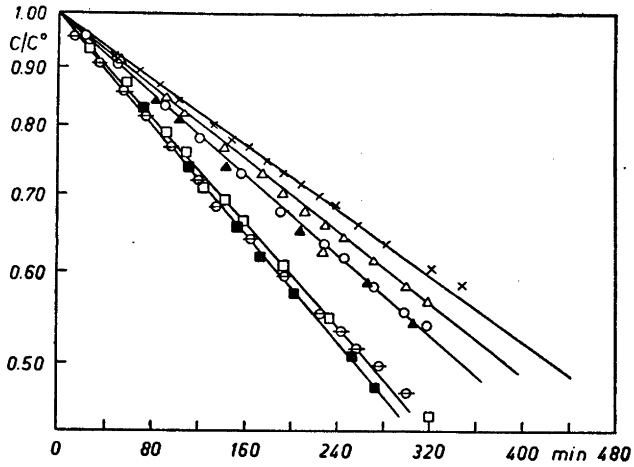

Fig. 5. Aquation of chloropentamminecobalt(III) at 52° C and pH = 1. ⊖, □, ■ — $[Co(NH_3)_5Cl]Cl_2$ *in* H_2O; △, ▲ — $[Co(ND_3)_5Cl]Cl_2$ *in* H_2O; × — $[Co(NH_3)_5Cl]Cl_2$ *in* D_2O; ○ — $[Co(ND_3)_5Cl]Cl_2$ *in* D_2O.

If exchange is slow, then the four differently substituted systems were in fact different, and, in principle, four aquation rates should be observable. This appeared to be the case, since half lives of 265, 365, 385, and 422 minutes were found. Comparisons between the last three should only be considered qualitatively, however, since these half lives are subject to an experimental error of perhaps five percent, in spite of precautions taken. The most conservative observation from the data is that the rate was reduced to some 65 percent of the normal value if deuterium was present *either* in the complex or in the solvent, or in both.

Aquation and hydrolysis of $[Co(NH_3)_5Br]Br_2$. A less detailed study was made of the aquation and hydrolysis of $[Co(NH_3)_5Br]^{+2}$ at 48° and 4.5°, respectively. In addition, the aquation rate in heavy water was determined, and found to be 75 % of that in ordinary water. The data are included in Table 4.

Some exploratory data were obtained on the aquation of $[Cr(NH_3)_5Cl]^{+2}$ and of $[Co(NH_3)_5(HCO_3)]^{+2}$, but the former was found to lose ammonia too rapidly to permit accurate rate studies, and the latter reaction was too fast, even at 0° C.

DISCUSSION

The rate constants obtained in this investigation are collected in Table 4, together with the available activation energy data, and comparisons with previous results. Where comparisons are possible, our results are in fair agreement with those of previous workers. In addition, from Garrick's value for the aquation rate of $[Co(NH_3)_5Cl]^{+2}$ at 25°, and ours at 52°, we calculate an activation energy of 22.9 Kcal/mole and an activation entropy of −9.0 cal/° mole (or E.U.).

Table 4. Summary of aquation and hydrolysis rate constants.

Aquation:
$[Co(NH_3)_5X]^{+2} + H_2O = [Co(NH_3)_5(H_2O)]^{+3} + X^-$

Complex	Solvent	$t°$ C	$k \times 10^3$ (min^{-1})		H (Kcal)	S (cal/° mole)
			this investig.	previous values		
$[Co(NH_3)_5Cl]^{+2}$	H_2O	25 52	2.53	0.100 [a]	22.9	−9.0
$[Co(ND_3)_5Cl]^{+2}$	H_2O	52	1.81			
$[Co(NH_3)_5Cl]^{+2}$	D_2O	52	1.65			
$[Co)ND_3)_5Cl]^{+2}$	D_2O	52	1.90			
$[Co(NH_3)_5Br]^{+2}$	H_2O	48	7.7	7.0 [b]	23.9 [b]	3.2
$[Co(NH_3)_5Br]^{+2}$	D_2O	48	5.6			

Hydrolysis:
$[Co(NH_3)_5X]^{+2} + OH^- = [Co(NH_3)_5(OH)]^{+2} + X^-$

Complex	Solvent	$t°$ C	$k_{2\text{-order}}$ (M^{-1} min^{-1})		H (Kcal)	S (cal/° mole)
			this investig.	previous values		
$[Co(NH_3)_5Cl]^{+2}$	H_2O	25 25.3 18.0 11.0 3.6 2.5	 51.6 15.2 4.48 1.09 0.91	78 [c]	28.7	37
$[Co(ND_3)_6Cl]^{+2}$	D_2O	18.0 2.5	8.6 0.57			
$[Co(NH_3)_5Br]^{+2}$	H_2O	25 15 4.5	 11.8	360 85 (15)	23.5	23

a) Ref.[22]; b) Ref.[9]; c) Ref.[10]; d) Ref.[9]

It is of interest first to compare the aquation and hydrolysis reactions for the all hydrogen system:

$$[Co(NH_3)_5Cl]^{+2} + H_2O = [Co(NH_3)_5(H_2O)]^{+3} + Cl^- \quad (7)$$
$$[Co(NH_3)_5Cl]^{+2} + OH^- = [Co(NH_3)_5(OH)]^{+2} + Cl^- \quad (8)$$

The thermodynamic ΔS_7 is −26.2 E.U., from data given by Latimer [19]. From J. Bjerrum [20], the entropy of dissociation of $[Co(NH_3)_5(H_2O)]^{+3}$ is *ca.* 6 E.U., which, in combination with the entropy of dissociation of water, leads to a value for ΔS_8 of −1.0. If, as has been argued, both aquation and hydro-

lysis proceed by an S_N2 mechanism [8], one would expect the entropies of activation to compare similarly with the overall entropies of reaction. This, however, is not the case, *viz.* -26.6 *versus* -9.0 and -1.6 *versus* 37.

In particular, it is difficult to account for the 39 E.U. difference between ΔS_8 and $\Delta S^{\ddagger}$ for the hydrolysis reaction, if an S_N2 process, going through a seven coordinated transition state, is truly involved. While there would be *ca.* 12 E.U. favoring this transition state because of the charge effect [21], there should be -8 E.U. from loss in translational entropy [22], plus a further negative term reflecting the smaller entropy of a seven coordinated structure as compared to a hexacoordinated one.

An alternative hydrolysis mechanism has been proposed by Garrick [12]:

$$[Co(NH_3)_5Cl]^{+2} + OH^- = [Co(NH_3)_4(NH_2)(Cl)]^{+1} + H_2O \text{ (rapid)} \qquad (9)$$

followed by a rate determining step in which the chloride group is replaced by water. The acid base pre-equilibrium (9) must, in fact, exist, and be rapid, in view of our exchange results. Furthermore ΔS_9, which would now contribute to the activation entropy, should be quite positive. Thus for

$$[Co(NH_3)_5(H_2O)]^{+3} + OH^- = [Co(NH_3)_5(OH)]^{+2} + H_2O \qquad (10)$$

ΔS_{10} is *ca.* 25 E.U., and ΔS_9 should be even larger because of the fifteen equivalent positions from which a proton can be removed, in spite of the adverse charge effect. In further support of this mechanism it should be noted that in the case of $[Co(CN)_5I]^{-3}$, where no acid base pre-equilibrium is possible, work in this laboratory shows the rate of appearance of free I^- to be independent of pH.

Actually, the principal evidence that cobalt(III) complexes may react by an S_N2 mechanism lies in the observation by Brown *et al.*[8] that substitution of Cl^- by N^{-3} in *cis* $[Co(en)_2Cl_2]^{+2}$ followed second order kinetics in methanol solution. However, this observation could equally well be explained in terms of the pre-equilibrium

$$[Co(en)_2Cl_2]^+ + X^- = [Co(en)_2Cl_2]^+ \; X^- \qquad (11)$$

followed by a rate determining exchange of X^- for Cl^-, whose molecularity is experimentally inaccessible.

Work in this laboratory has shown that the absorption spectrum of $[Co(en)_2Cl_2]^+$ in methanol, in the presence of N_3^-, shows increased absorption in the near ultra violet, an effect similar to that found by Evans and Nancollas [23] in their study of ion pair formation between $[Co(NH_3)_6]^{+3}$ and halide ions in aqueous media. A similar situation prevailed with I^-, which we found also to give second order substitution kinetics in methanol. For N_3^- and I^-, ion pair formation constants of *ca.* 10 and *ca.* 20 were indicated. That such ion pair formation can indeed be a kinetic precourser to substitution has been shown by Taube and Posey [24].

Proceeding to the matter of rates in deuterated systems, several effects have been found to contribute to changes in rate when hydrogen is replaced by deuterium. First, there is a general solvent effect, which is small. The solubilities of salts per mole of water are about ten percent less in D_2O[25], and activity coefficient ratios are nearly invariant [26]. In addition, reactions for which the

only effect on rate should be that of changing solvent environment, show only slight differences in velocity in the two media. Thus the rates of solvolysis of methyl halides were unchanged in D_2O[15].

Interestingly, however, these last authors also reported that the rate of acid hydrolysis of tertiary butyl chloride was some 40 % slower in D_2O. This, however, they correlated with the high dependence of the reaction upon electrophilic solvation. Thus the rate of this reaction is 340 000 times faster in water than in methanol, while that of methyl bromide is only 27 times faster. Since aquation and hydrolysis reactions for cobalt complexes are generally only about 10 fold slower in alcoholic media than in water, this type of solvent effect should not be important here.

An important effect, in relation to the present studies, arises when an acid base pre-equilibrium exists. A number of acid and base catalyzed reactions have been studied in D_2O, with the observation that the rates may range from being twice as fast to being twice as slow as in H_2O[25]. A bond between deuterium and another atom is generally stronger than the corresponding one with hydrogen, due to the zero point energy effect [27]. Thus the dissociation constants of weak acids and bases are several fold less in D_2O [13], and the O—D—O bond in the acetic acid dimer may be somewhat weaker than the corresponding O—H—O bond [28].

All of the reactions studies in the present investigations were slower in deuterium containing systems, and the differences, being of the order of 30 to 60 %, were greater than to be expected from general solvent effects. The 55 to 60 % decrease in rate of hydrolysis is certainly best explained as arising from the acid base pre-equilibrium postulated above.

The isotope effects on aquation rates require more explanation, however. They do not seem to be accountable for by either an ordinary S_N1 or S_N2 mechanism. In the former case, one would not expect a reduced rate of aquation for the H-complex in D_2O, and for the latter, the rate for the D-complex in H_2O should be normal. It seems possible, however, to explain the various aspects of the aquation reaction if one supposes that the ammine complexes are extensively hydrogen bonded with solvent, and that the water molecules so involved play a specific role in directing the course of the reaction. The diagram below illustrates how water molecules might reasonably form bridges between the ammonia groups and the chloride ion in $[Co(NH_3)_5Cl]^{+2}$. For purposes of clarity, only two of the four bridges are shown. The second dia-

gram is to scale, assuming 2.6 A° for the N—H—O bond, and tetrahedral angles on the nitrogen and oxygen. The energy of the O—H—Cl interaction is perhaps several kilocalories, judging from the case of *o*-chlorophenol [29]. This

partial solvation of the chloride should aid in its removal during the aquation step, which is pictured as a concerted motion of the chloride outward, and one water molecule inward. Since most of the −26 E.U. entropy for the aquation reaction is ascribable to the charge effect, the more positive entropy of activation, −9 E.U., is explainable if, in the transition state, the Co—Cl distance is only slightly greater than normal. With deuterium present, *either* on the nitrogen or the oxygen, the bridge may be somewhat weaker. Thus replacement of a proton by a deuteron in either the N—H—O or the O—H—Cl position might be expected to give similar results, as is observed.

One might expect, however, that if both hydrogens were replaced by deuterium, an even slower rate of aquation should result, in seeming contradiction with our findings. The experiment with D-complex in D_2O, however, was not conceived with the picture of four equivalent sets of hydrogen bridges in mind, and the percent deuteration was only 85 to 90. Under this circumstance, there was only a minority chance that all *four* of the bridges be deuterated at *both* positions, and the aquation could take place primarily through a singly deuterated bridge.

As final points, the above representation accounts nicely for the stereospecificity of the aquation process, such as Mathieu's observation [30] that $[Co(en)_2NH_3Br]^{+2}$ underwent mutarotation to the corresponding monoaquo species without racemization and with retention of configuration. Also, the explanation previously given [5] for the specific anion effects on the aquation rates of Co(III) acidopentammines would still apply. The observed decrease [6] in the rate of aquation with decreasing number of N—H in cobalt(III) complexes is consistant with the proposed hydrogen bonding picture.

The above conclusions allow a distinction to be made between alternative mechanisms for the hydrolysis reaction. The complete mechanism may be pictured as consisting of reaction (9), followed by either

$$[Co(NH_3)_5(NH_2)Cl]^+ + H_2O = [Co(NH_3)_5(OH)]^{+2} + Cl^- \quad \text{(slow)} \qquad (12)$$

or

$$[Co(NH_3)_4(NH_2)Cl]^+ \rightarrow [Co(NH_3)_4(=NH_2)]^{+2} + Cl^- \quad \text{(slow)} \qquad (13)$$

$$[Co(NH_3)_4(=NH_2)]^{+2} + H_2O \rightarrow [Co(NH_3)_5OH]^{+2} \quad \text{(fast)} \qquad (14)$$

as rate determining steps. Reaction (12), an aquation type step, would not account for the low stereospecificity of hydrolysis reactions. These are generally accompanied by isomerization in contrast to aquation which takes place with retention of configuration. In the alternative sequence proposed, loss in configuration would well occur if there is a pentacoordinated intermediate, stabilized by double bonding to the amide group. It has been suggested, moreover, that such double bonding stabilization may account for the accelerating effect of NH_2^- and OH^- groups, relative to other negative groups, on the aquation rates of Co(III) complexes [31].

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Utilization of Deoxyuridine and 5-Methyluridine for the Biosynthesis of Thymine by the Rat

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5-Methyluridine and deoxyuridine were prepared from thymine-2-^{14}C and uracil-2-^{14}C, respectively, by the action of a nucleoside phosphorylase from an extract of lyophilized *Escherichia coli*. The compounds were identified by light absorption curves, sugar reactions and titration with metaperiodate.

The two labeled nucleosides were injected into partially hepatectomized rats and their utilization for pyrimidine synthesis in the liver and intestine was studied. The incorporation data were compared with values from parallel experiments with ^{14}C labeled uracil, uridine, uridine-5′-phosphate, thymine, thymidine and orotic acid.

Uracil showed a very small incorporation into PNA pyrimidines and DNA thymine. Uridine was utilized for all pyrimidines, the amount of isotope incorporation increasing rapidly with higher dose levels. Thymine and 5-methyluridine were slightly utilized for synthesis of DNA thymine in regenerating liver.

Deoxyuridine was in the liver and intestine incorporated into DNA thymine and to a much smaller extent into PNA uracil from intestine. In agreement with earlier experiments thymidine was exclusively incorporated into DNA thymine.

It is concluded that the ribose of uridine (or uridine-phosphate) is first reduced to deoxyribose forming deoxyuridine (phosphate) which accepts a methyl group to form thymidine (phosphate).

The free pyrimidine bases uracil, cytosine and thymine are very poorly or not at all utilized for the biosynthesis of polynucleotide pyrimidines by the rat [1-5]. Cytidine and to a smaller extent uridine, however, are readily utilized for the synthesis of both PNA * and DNA * pyrimidines [6], as are deoxycytidine and thymidine for DNA pyrimidines [7]. From the incorporation of the ^{15}N labeled ribosides into DNA pyrimidines in contrast to the metabolic inertness of the corresponding free pyrimidines we came to the conclusion that the rat is able to convert pyrimidine-attached ribose to deoxyribose without rupture of the glycosidic linkage [6]. Using cytidine-^{14}C labeled in both the base

* The following abbrevations are used in this paper: Pentosenucleic acid, PNA; Deoxypentosenucleic acid, DNA; Uridine-5′-phosphate, UMP; Adenosine triphosphate, ATP.

and ribose Rose and Schweigert [8] demonstrated the intact incorporation of this nucleoside into PNA and DNA pyrimidine nucleosides and thus definitively established a reaction of this type.

The present work represents a first attempt to find possible intermediates in the conversion of a riboside to a deoxyriboside. With the aid of a pyrimidine nucleoside phosphorylase from an extract of *E. coli* [9, 10] it was possible to synthesize labeled deoxyuridine from uracil-2-^{14}C + deoxyribose-1-phosphate and labeled 5-methyluridine from thymine-2-^{14}C + ribose-1-phosphate. The incorporations of these compounds into the polynucleotides from regenerating liver and intestine were studied at different dose levels and compared with corresponding incorporation experiments using labeled thymine, thymidine, uracil, uridine, UMP and orotic acid.

MATERIALS AND METHODS

Uracil-2-^{14}C * was synthesized according to Johnson and Flint [11], *orotic acid-2-^{14}C* according to Nyc and Mitchell [12], *thymine-2-^{14}C* according to Scherp [13].

Ribose-1-phosphate was prepared as the di*cyclo*hexylammonium salt according to Klenow [14], *deoxyribose-1-phosphate* according to Friedkin and Roberts [15]. In the latter case the phosphorylase from *E. coli* was used instead of Friedkin's liver enzyme.

For the preparation of the nucleosides the *nucleoside phosphorylase* activity from *E. coli* [9, 10] was used. A cell free extract of the lyophilized bacteria was prepared as described earlier [16]. The extract was diluted with an equal volume of water, precipitated in the cold with one tenth volume of M $MnCl_2$ and allowed to stand for 30 minutes at 0° in order to remove nucleic acids. After centrifugation the clear supernatant was dialyzed for four hours against a total of four liters of 0.01 M phosphate buffer, pH 7.0 and then lyophilized. The dry powder was stored at −15° in a desiccator without appreciable loss of activity for several weeks.

Chromatographic separation of free pyrimidines from corresponding ribosides or deoxyribosides. For this purpose ion exchange on Dowex-2, formate (200−400 mesh) was used. A column of 2 cm diameter and 15 cm length was sufficient for the separation of 50−500 μmoles of pyrimidine compounds. A 4 × 15 cm column was used for larger amounts. Before starting the chromatogram the pH of the solution was adjusted to *ca.* 10 by addition of concentrated ammonia. The solution was introduced to the column by gravity and elution carried out with ammonium formate, pH 9.0 (formate concentration = 0.1 M). The pyrimidine nucleoside or deoxynucleoside emerged between 4 and 7 column volumes, immediately followed by the free base. This method satisfactorily separated nucleosides from the corresponding free bases, but did not resolve either the nucleosides or the free pyrimidines.

Fractions containing the same pyrimidine compound were combined and after evaporation to dryness *in vacuo* passed through a Dowex-50 column (H^+ form, 2 × 10 cm) to remove ammonia. The column was washed with water until the effluent was free from light absorption at 260 mμ. Formic acid was removed from the effluent by repeated evaporation *in vacuo*. The dry residue was dissolved in water and used directly for the experiments.

Further resolution was attained by a combination of this method and chromatography on starch columns with water saturated butanol [17]. In this way a mixture of thymine, thymidine, uracil and deoxyuridine was separated. First pyrimidines were separated from deoxyribosides by ion exchange chromatography. Subsequently the two compounds in each group were separated by starch chromatography. Under these conditions deoxyuridine had an *R* value of 0.81.

Starch chromatography with butanol-water also allowed direct separation of thymine from 5-methyluridine. The *R*-value for the latter compound was 0.60 (thymine = 1.48).

* We wish to thank Dr. H. L. Smith for the synthesis of the labeled uracil.

Preparation of bases from polynucleotides. PNA and DNA were prepared and separated according to Hammarsten [18]. DNA was hydrolyzed at 175° with formic acid [19] and the bases separated by chromatography on Dowex 50 [20, 21]. Since thymine was eluted in the front with water, contamination was possible. In several cases it was therefore rechromatographed on starch [17]. However, the specific activity of the thymine before and after starch chromatography never showed significant differences.

The PNA nucleotides were hydrolyzed with N HCl for one hour at 100°. After removal of HCl and adjustment of pH to 9—10 the mixture of purines and pyrimidine nucleotides was chromatographed on a Dowex-2-chloride column (diameter 0.9 cm, length 15 cm) according to the principles of Cohn [22]. Elution was started with 0.0025 N HCl. Two peaks showing absorption around 260 mμ were obtained. The first consisted of a mixture of adenine and guanine; the second was cytidylic acid. After elution of the cytidylic acid peak, the uridylic acid was eluted with 0.02 N HCl. In one experiment a separation of PNA purines was desired. This was achieved by chromatography on Dowex-50 (H^+ form, solvent 3 N HCl) after the Dowex-2 chromatogram.

Measurement of radioactivity. Fractions containing the same purine or pyrimidine compound were combined after chromatography and the acid removed by repeated evaporation *in vacuo*. The residue was dissolved in 5 ml of water. Aliquots were diluted with 0.05 M phosphate buffer, pH 7.2, for light absorption measurements. From the absorption at 260 mμ the concentration of each compound was calculated [23, 24]. Radioactivity measurements were carried out in a Tracerlab Sc-18 windowless flow counter on infinitely thin samples (*ca.* 1 μmole of compound) of non diluted aliquots.

RESULTS

Enzymic synthesis of labeled nucleosides

5-Methyluridine. A typical preparation is given below. Thymine-2-^{14}C (30 mg), dissolved in 5 ml of 0.05 M phosphate buffer, pH 7.0, was added to 63 mg (125 μmoles) of di*cyclo*hexylammonium ribose-1-phosphate (85 % pure) and 40 mg of lyophilized enzyme. Incubation was carried out at 37°. In preliminary experiments it had been found that better yields of riboside were obtained in phosphate than in tris buffer, probably because phosphate inhibits breakdown of ribose-1-phosphate [10].

The reaction was stopped by immersion in boiling water for one minute when there was no further decrease in light absorption at 300 mμ. The light absorption was measured on aliquots of the reaction mixture diluted with 0.1 N NaOH (*cf.* Ref. [15]). Usually the reaction was complete after 4 hours. Separation of the nucleoside from the remaining thymine was carried out by ion exchange chromatography. 80 μmoles of riboside (yield *ca.* 60 % based on ribose) and 130 μmoles of thymine were recovered.

Deoxyuridine. This deoxynucleoside was prepared by two different methods. One way consisted of incubation of uracil-2-^{14}C with deoxyribose-1-phosphate and enzyme in the manner described above for 5-methyluridine. The yield based on deoxyribose was 40—50 %. In the second method deoxyribose-1-phosphate was not isolated, but was formed and reacted by the following reactions:

Thymidine + phosphate ⇌ deoxyribose-1-phosphate + thymine
Deoxyribose-1-phosphate + uracil-2-^{14}C ⇌ deoxyuridine-2-^{14}C + phosphate

Uracil-2-^{14}C (400 μmoles) was dissolved in 15 ml of 0.05 M phosphate buffer, pH 7.0. Thymidine (400 μmoles) and 150 mg of enzyme were added and incubation was carried out at 37° for 6 hours. By a combination of ion

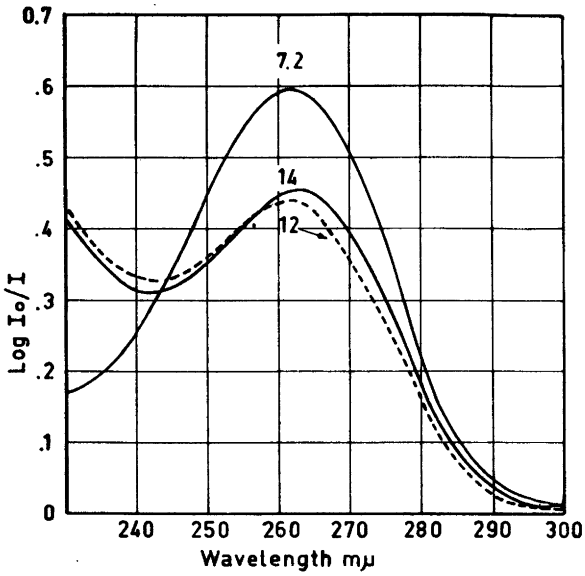

Fig. 1. Ultra violet absorption of deoxyuridine in aqueous solution at pH values indicated.

exchange and starch chromatography deoxyuridine (120 μmoles), uracil, thymidine and thymine were separated.

The second method was less time consuming than the first one and even though the yield of deoxyuridine was lower (*ca.* 30 %) it was used when larger amounts were prepared. The recovery of nonreacted isotopic uracil was almost quantitative.

Thymidine. This deoxyriboside was prepared by incubation of thymine-2-^{14}C (200 μmoles, 140 000 ct/min/μmole) with nonlabeled thymidine (100 μmoles) and 50 mg of enzyme in 5 ml of 0.1 M phosphate buffer, pH 7.0, for 5 hours. The thymidine-^{14}C formed during the exchange reaction was isolated by ion exchange chromatography. It amounted to 86 μmoles and had a specific radioactivity of 80 000 ct/min./μmole.

Uridine. Our enzyme preparation from *E. coli* had a surprisingly low phosphorylytic activity towards uridine. Labeled uridine was therefore prepared from orotic acid-2-^{14}C *via* UMP. The enzymic synthesis of UMP was carried out in a crude extract of rat liver from ribose-5-phosphate, ATP, and orotic acid as described earlier [25]. The protein free perchloric acid supernatant was hydrolyzed for 1 hour at 100° to break pyrophosphate linkages. UMP was purified by chromatography with formic acid on Dowex-2 [26]. After removal of the formic acid UMP was dephosphorylated with snake venom phosphatase [26] and the resulting uridine was then chromatographed as described in the experimental part.

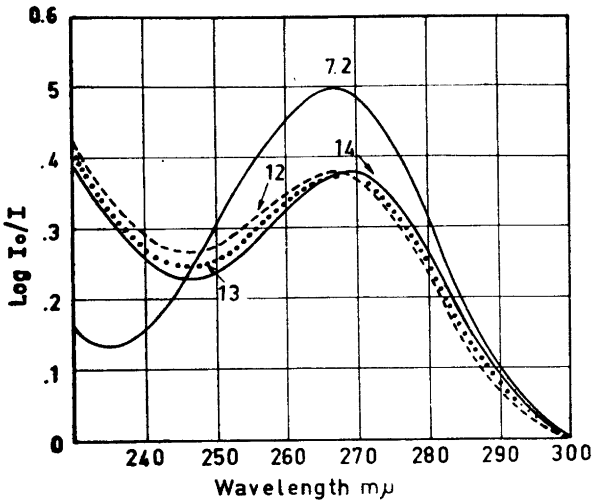

Fig. 2. Ultra violet absorption of 5-methyluridine in aqueous solution at pH values indicated.

Characterization of 5-methyluridine and deoxyuridine

Ultra violet curves. Light absorption curves of the compounds were measured at different pH in a Beckman Spectrophotometer, model DU (Figs. 1 and 2). Since the compounds were not crystalline values for log I_0/I are given rather than molecular extinction coefficients *. The curves for deoxyuridine (Fig. 1) show the same maxima and minima and the same isosbestic points as given by Fox and Shugar [27] for deoxyuridine obtained by chemical deamination of deoxycytidine.

No similar light absorption curves for 5-methyluridine have been found in the literature. The extensive studies of Fox and Shugar [24,27] have demonstrated that the nature of the sugar attached to a pyrimidine in nucleosidic linkage influences in certain systematic ways the light absorption of the pyrimidine, especially in the alkaline range. Thus according to the predictions by the cited authors 5-methyluridine and uridine would be expected to show similar changes at pH 12—14 (reflecting dissociation of sugar hydroxyls). The presence of the methyl group on the pyrimidine ring would largely result in a bathochromic shift of the curves.

The curves of Fig. 2 fulfil these conditions. The first isosbestic point at 2 475 Å is identical with that of thymidine and represents the dissociation of the 6-OH-group. This point indicates that the compound, in analogy with thymidine, has a furanose structure, since thymine pyranosides show an isos-

* The molar extinction coefficients could be calculated from nitrogen analyses of the solutions which were used for light absorption measurements. Under those circumstances values of 9 800 (at 267 mμ and pH 7.2) and 10 200 (at 262 mμ and pH 7.2) were obtained for 5-methyluridine and deoxyuridine respectively.

bestic point at shorter wave lengths [27]. In comparison to thymidine [27] the sugar dissociation of the compound starts at lower pH, as indicated by the difference between the pH 12 and pH 13 curves. Furthermore, the shift of the isosbestic point from 2 475 Å to 2 685 Å is larger than the corresponding shift for thymidine (2 475 Å to 2 640 Å). Both of these results show that the compound is a riboside and not a deoxyriboside.

The light absorption data are thus in full accordance with a formulation of the compound as 5-methyluracil-ribofuranoside.

Sugar reactions. 5-Methyluridine gave a positive orcinol test for ribose [28]. Color development was not complete after two hours and was considerably lower than that of adenosine. Parallel experiments with uridine showed similar results.

When deoxyuridine was compared with thymidine in the cysteine-sulfuric acid test for deoxyribose [29] it was found that deoxyuridine after one hour showed 83 % of the amount of color obtained from an equimolar amount of thymidine.

Paper chromatography. Each ^{14}C compound used in the animal experiments showed a single radioactive and light absorbing (Mineralite lamp) spot on paper in all the tested solvents. A summary of the different R_F values is given in Table 1.

Table 1. R_F values of ^{14}C-pyrimidines and nucleosides. The spots were localized by radioactivity and by ultra violet absorption (except in the pyridine containing solvent).

	Butanol : water 87 : 13	Butanol : conc. NH_3 75 : 25	*t*-butanol : pyridine : water 65 : 25 : 15
Uracil	0.34	0.20	0.60
Thymine	0.49	0.41	0.67
Uridine	0.17	0.07	
Thymidine	0.48	0.41	0.74
Deoxyuridine	0.34	0.15	0.71
5-Methyluridine	0.31	0.20	0.66

Metaperiodate titration. The furanose structure of the nucleosides was established by titration with metaperiodate as described by Manson and Lampen [30]. Deoxyuridine did not consume any periodate during 24 hours. 5-Methyluridine (21 μmoles) consumed 20.2 μmoles of periodate under the same conditions.

Incorporation experiments

Thymine derivatives. White albino rats weighing 150—155 g each were subjected to partial hepatectomy by the technique used in this laboratory [31]. Each isotopic compound was dissolved in a total of 4 ml of physiological sodium chloride and injected subcutaneously in the back of a single rat 24 and 26 hours after the operation. Five hours after the last injection the animal was killed. The liver and the thoroughly washed small intestine were immediately removed and put into alcohol.

The pyrimidines of the polynucleotides from the regenerating liver and the small intestine were prepared and analyzed for ^{14}C with the results shown in Table 2. The purines are not included in the table since all analyses here and in the experiments with uracil derivatives showed insignificant amounts of isotope. Similar experiments with orotic acid are also included in the table for comparison.

Table 2. Incorporation of thymine-^{14}C compounds and orotic acid-^{14}C into regenerating liver and intestine. Specific activities of precursors (count/min/μmole): thymine: 140 000; thymidine: 14 000; 5-methyluridine: 140 000; orotic acid: 310 000. The values in the table are given for a specific activity of 100 000 count/min/μmole in the precursors.

Isolated	Precursor	thymine		thymidine		5-methyl-uridine		orotic acid	
	μmoles injected	6	40	6	40	6	40	6	40
Liver	*PNA:* uridylic acid	5	15	0	0	15	15	1 600	6 500
	cytidylic acid	5	7	25	0	0	10	600	5 200
	DNA: thymine	30	340	290	7 500	70	380	600	1 500
	cytosine	10	25	25	0	5	0	420	1 000
Intestine	*PNA:* uridylic acid	0	20	30	—	15	20	45	380
	cytidylic acid	0	15	10	5	0	10	35	270
	DNA: thymine	5	15	2 200	4 400	20	0	45	230
	cytosine	0	7	0	25	0	0	25	100

Thymidine was a very efficient precursor for DNA thymine in both liver and intestine. Thymine and 5-methyluridine showed a similar behaviour in that both were utilized to a significant extent for the synthesis of DNA thymine by liver but not by the intestine. Orotic acid was well utilized for the synthesis of all liver pyrimidines. The same general pattern was obtained in the intestine, although the amount of ^{14}C incorporation there was much smaller.

Uracil derivatives. Table 3 shows similar experiments with different uracil containing compounds. Free uracil showed a small but significant incorporation into PNA uracil in some experiments. There was a still smaller utilization of uracil for PNA cytosine and DNA thymine. Uridine was significantly incorporated into all pyrimidines at doses of 40 and 100 μmoles, with maximum incorporation into PNA uracil. Deoxyuridine was found to be well utilized for DNA thymine synthesis by both liver and intestine. In the intestine it was also slightly incorporated into PNA uracil. Uridine-5-phosphate was not significantly utilized for pyrimidine synthesis in the liver, but was somewhat more efficient than uridine for PNA uracil synthesis in the intestine.

Table 3. Incorporation of uracil-^{14}C compounds into regenerating liver and intestine. Specific activities of precursors (count/min/μmole): Uracil: 98 000 ct.; uridine: 62 000; deoxyuridine; 98 000; UMP: 38 000. The values in the table are given for a specific activity of 100 000 count/min/μmole in the precursor.

Isolated	Precursor	uracil		uridine			deoxy-uridine		UMP
	μmoles injected	40	100	6	40	100	6	40	40
Liver	*PNA:* uridylic acid	10,0	100	10	150	250	3	25	30
	cytidylic acid	20,0	60	1	50	170	0	15	0
	DNA: thymine	10,30	60	15	70	330	180	530	20
	cytosine	3,10	0	3	50	130	10	0	0
Intestine	*PNA:* uridylic acid	−,25	80	15	200	440	60	90	400
	cytidylic acid	30,3	50	10	130	180	10	10	30
	DNA: thymine	40,15	40	0	20	160	185	680	30
	cytosine	40,3	0	0	30	130	5	10	30

One rat received a total of 100 μmoles of deoxyuridine-^{14}C divided into 6 doses during 3 days. Incorporation in the intestine was again limited to DNA thymine (and PNA uracil) as shown in Table 4.

DISCUSSION

The first consideration which must be discussed is that of the chemical nature and purity of the enzymically synthesized nucleosides. Crystallization of the substances was not carried out and elementary analyses and melting points are therefore not available. Identification of the compounds as 5-methyluridine and deoxyuridine rests on the following points: (a) sugar reactions, which show the presence of ribose and deoxyribose respectively, (b) metaperiodate titration, which showed that the compounds had a furanose structure, (c) light absorption data at different pH and, (d) the mode of synthesis.

Table 4. Incorporation of deoxyuridine-^{14}C (98 000 ct/min./μmole) into intestinal nucleic acids of a non-hepatectomized rat.

		specific activity (ct/min./μmole)
PNA:	adenine	0
	guanine	0
	uridylic acid	70
	cytidylic acid	30
DNA:	adenine	0
	guanine	0
	thymine	2 300
	cytosine	20

In addition to the rate of polynucleotide synthesis the relative incorporation of the various isotopic compounds might be determined by other uncontrolled effects such as the permeability of the cells for the precursor, the size of the "precursor pool" in the body and the presence of competing processes which keep the precursor from reaching the site within the cell where it is utilized for polynucleotide synthesis. In the case of the nucleosides this last factor may have played a very important role because of the high concentration of nucleoside phosphorylases [32] and possibly hydrolases in most organs. The demonstrated specificity of some of these enzymes for uridine might possibly explain the lower incorporation of this nucleoside in comparison to cytidine [6]. The possibility of the metabolic inhomogeneity of the polynucleotides [33] introduces one more reason why great caution must be exercised when interpreting the "effectiveness" of different precursors.

A general idea of the extent to which some of these factors influence the observed amounts of incorporation might be obtained by comparing the utilization of a precursor at different dose levels. This was done in the present investigation not with the purpose to gain information about rates of polynucleotide biosynthesis but in order to find out if the administered precursor could be considered as an intermediate in the conversion of ribose to deoxyribose derivatives. One reason why these incorporation experiments do not measure rates of pyrimidine synthesis is that one cannot decide, whether a net synthesis or an exchange reaction is observed.

The results with the *thymine-^{14}C compounds* (Table 2) confirmed the earlier demonstrated specific incorporation of thymidine-^{15}N into DNA thymine. Thymidine-^{14}C incorporation was of the same order of magnitude as that of orotic acid in the liver, while in the intestine orotic acid incorporation was much smaller. This was largely the result of the low utilization of orotic acid in extra hepatic organs in experiments of relatively short durance.[34]

Thymine and 5-methyluridine showed the same general incorporation pattern. In confirmation of the finding of Holmes *et al.* [5] some utilization of thymine-^{14}C was found for DNA thymine biosynthesis in regenerating liver (but not in the intestine), especially at the higher dose level. The similarity of the results between the two substances makes it seem likely that 5-methyluridine was incorporated after breakdown to free thymine.

All *uracil-^{14}C compounds* were incorporated to some extent. In some experiments free uracil was significantly incorporated into PNA pyrimidines and into DNA thymine. The incorporation was very small and the possibility exists that it occurred after breakdown to CO_2. In all these experiments, however, the purines showed no significant incorporation of isotope, which is evidence for a specific incorporation of the labeled uracil.

Uridine was incorporated at the two higher dose levels into all polynucleotide pyrimidines five to ten times more effectively than free uracil. The mechanism of uridine incorporation did not therefore involve conversion to free uracil. The results were closely dependent on the amount of uridine injected, and at the lowest dose level no incorporation could be observed. This and the corresponding result with thymidine incorporation in liver might be explained by the action of nucleoside phosphorylase on the injected precursors. At low levels the nucleosides might have been almost completely split

by the enzyme and therefore were not available for polynucleotide synthesis. At higher doses increasing amounts of the injected nucleoside would be available for synthetic processes. These considerations make impossible direct comparison of nucleoside incorporation as was done earlier with uridine and cytidine [6].

UMP was slightly more effectively incorporated than uridine in the intestine, but incorporation in the liver was very small. In this case the well known low permeability of nucleotides might explain the low incorporation. It is not possible therefore to conclude from *in vivo* experiments whether the incorporation of uridine takes place via uridine-5-phosphate.

A type of incorporation was found with deoxyuridine which was similar to that of thymidine rather than uridine. It was almost exclusively incorporated into DNA thymine both in regenerating liver and intestine. A small but significant conversion to PNA uracil was observed in the intestine but not in liver.

The incorporation data are in accordance with the view that uridine (or a derivative of uridine) is transformed by the rat to DNA thymine and that deoxyuridine (or derivative) and thymidine (or derivative) are intermediates in this process. The first step involves a reduction at C_2 of ribose while the second step consists of the attachment of a methyl group to the uracil ring. Deoxyuridine might also arise through deamination of deoxycytidine. The possibility of phosphorylated derivatives as intermediates for these reactions rather than nucleosides must be strongly considered. In liver preparations uridine is quite rapidly phosphorylated to UMP in the presence of ATP *. Phosphorylated intermediates rather than nucleosides are furthermore indicated in pyrimidine biosynthesis from orotic acid [35, 25] and in purine biosynthesis [36, 37]. This question can be answered only from experiments at the enzyme level.

An attempt to summarize the available incorporation data is made in Fig. 3. It is probable that the diagram will have to be modified by future results,

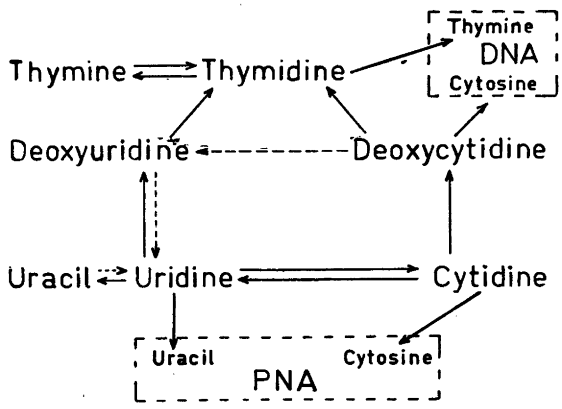

Fig. 3. Possible interrelations between nucleosides in the rat.

* Unpublished experiments.

especially with respect to the level of phosphorylation of the intermediates involved.

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Note added in proof. Friedkin and Roberts (*Federation Proc.* **14** (1955) 215) have recently briefly described the incorporation of deoxyuridine into DNA thymine from suspensions of chick embryo and bone marrow. These observations are in accordance with the intermediate position of deoxyuridine in DNA synthesis as pictured in Fig. 3.

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Bond Refractions, Bond Dispersions and Ring Refractions in *Cyclo*polymethylenesilanes

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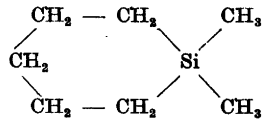
Bond refractions of the *cyclo*trimethylene, *cyclo*tetramethylene and *cyclo*pentamethylene groups bonded to silicon have been calculated. Bond dispersions of the last-mentioned group are also given. It is shown that a ring refraction is present in the *cyclo*trimethylene- and *cyclo*tetramethylenesilane rings.

Reliable refractometric data for *cyclo*polymethylenesilanes were first given by Bygdén [1] in 1915 for *cyclo*pentamethylenedichlorosilane and the corresponding dimethylsilane. Some years ago the present author [2] measured some refractometric constants of *cyclo*pentamethylenediethoxysilane. Recently West [3] reported some preparations of *cyclo*polymethylenesilanes with 4, 5 and 6 methylene groups in the heterocyclic ring, together with their refractometric constants. At about the same time Sommer and Baum [4] succeeded in preparing *cyclo*trimethylenedimethylsilane. With the accumulation of these data it seems justified to calculate the bond refractions, bond dispersions and ring refractions of some polymethylene groups bonded to silicon.

The material used in these calculations is listed in Tables 1 and 2. The structural type of the *cyclo*polymethylenesilanes is apparent from the following formula for *cyclo*pentamethylenedimethylsilane.



Bond refractions and bond dispersions. In the calculation of the bond refractions r [Si $(CH_2)_n$] the principles previously outlined by the author were applied [2].

Table 1. Molar refractions and molar dispersions of cyclo*pentamethylenesilanes.*

No.	Compound	MR_C	MR_D	MR_F	MR_g*	MR_{F-C}	MR_{g-C}	Ref.
1	$(CH_2)_5Si(CH_3)_2$	41.80	42.01	42.54	42.95	0.740	1.155	1
2	$(CH_2)_5Si(OC_2H_5)_2$	52.49	52.74	53.32	53.77	0.830	1.282	2

* Bygdén measured MR_G. The value of MR_g was obtained using the "reciprocal plotting method" of Bauer and Fajans [5].

Table 2. Molar refractions of cyclo*polymethylenesilanes.*

No.	Compound	MR_D	Ref.
3	$(CH_2)_5Si(OCH_3)_2$	43.31	3
4	$(CH_2)_5Si(CH_3)_5$	53.77	3
5	$(CH_2)_5Si(CH_2)_4$	49.27	3
6	$(CH_2)_4Si(CH_2)_4$	44.80	3
7	$(CH_2)_4Si(OCH_3)_2$	38.82	3
8	$(CH_2)_3Si(CH_3)_2$	33.22	4
9	$(CH_2)_6Si(CH_3)_2$	47.45	3

The normal bond refraction* r [Si $(CH_2)_5$] is obtained from the molar refraction of compound No. 4, for example, by dividing it by two or from the molar refraction of compound No. 1 by subtraction of $2r$ (Si—CH_3) **, assuming additivity of the bond refractions of the *cyclo*pentamethylene group and the two methyl groups to be in hand. The fact that the same value of r [Si $(CH_2)_5$] is obtained in both cases shows this assumption to be correct. The refractometric equivalence of a *cyclo*pentamethylene group with two alkyl groups when bonded to silicon is further demonstrated by the calculation of r [Si $(CH_2)_5$] from the molar refractions of the two *cyclo*pentamethylene-dialkoxysilanes, Nos. 2 and 3. As shown previously [2] the molar refractions of a dialkyldialkoxysilane may be obtained from the normal bond refractions of the alkyl and alkoxy groups bonded to the silicon atom using the formula

$$2r\,(\text{Si—R}) + 2r\,(\text{Si—OR}) - 2K_r^{***} = MR \qquad (1)$$

* The bond refraction of a group X bonded to silicon is written r (Si—X) and means the sum of the refractions of the group X and the bond Si—X. The normal value of the bond refraction r (Si—X) is defined as the bond refraction of the group in the compound SiX_n and is obtained by dividing the molar refraction by n. Usually n equals 4, but in the present case its value is 2. Thus the normal bond refraction of the *cyclo*pentamethylene group bonded to silicon is, by definition, equal to the molar refraction of bis-(*cyclo*pentamethylene)-silane (No. 4) divided by two.

** The bond refractions, bond dispersions and structure corrections used in the derivation of the bond values and ring refractions in this work were, unless otherwise stated, taken from Ref.[2]

*** K_r is named "the structure correction for refraction" and is used in silanes which at the same time have alkyl and alkoxy or siloxy groups bonded to the silicon atom (*cf.* Ref.[2]). The same applies to the structure correction for dispersion K_D, which is used in the following discussion.

Assuming the same eq. to be valid for a *cyclo*pentamethylenedialkoxysilane we get

$$r\,[\mathrm{Si}\,(\mathrm{CH_2})_5] + 2r\,(\mathrm{Si—OR}) - 2K_r = MR \qquad (2)$$

When substituting the appropriate values of r (Si—OR) and K_r and of MR from Table 1, r [Si $(CH_2)_5$] is obtained. Within the limits of error the same values are arrived at as from the molar refractions of compounds Nos. 1 and 4.

The refraction of the *cyclo*tetramethylene group bonded to silicon may be calculated in the same way as described for the *cyclo*pentamethylene group using the molar refractions of compounds Nos. 6 and 7. Eq. 2 applies to *cyclo*tetramethylenedimethoxysilane (No. 7) as well, if r [Si $(CH_2)_4$] is substituted for r [Si $(CH_2)_5$]. From the value of the molar refraction of the spirane, *cyclo*tetramethylene-*cyclo*pentamethylenesilane (No. 5), the additivity of the bond refractions of the two *cyclo*polymethylene groups is apparent.

For the calculation of the bond refraction of the *cyclo*trimethylene group bonded to silicon only the molar refraction (D-line) of *cyclo*trimethylenedimethylsilane (No. 8) is available.

The material on *cyclo*hexamethylenesilanes includes only the molar refraction (D-line) of *cyclo*hexamethylenedimethylsilane (No. 9). As pointed out below there is some doubt as to the accuracy of this value. On that account the calculation of the bond refraction of the *cyclo*hexamethylene group is postponed until further experimental results are available.

In the calculation of the bond dispersions of the *cyclo*pentamethylene group bonded to silicon, d [Si $(CH_2)_5$] *, the same methods were used as for the bond refractions. By subtraction of $2d$(Si—CH_3) from the molar dispersion of compound No. 1 one value of d[Si $(CH_2)_5$] is obtained; another results from the molar dispersion of compound No. 2. In this case we get

$$d\,[\mathrm{Si}\,(\mathrm{CH_2})_5] + 2d\,(\mathrm{Si—OC_2H_5}) - 2K_d = MR_{\lambda_1-\lambda_2} \qquad (3)$$

where K_d is the structure correction for dispersion. As yet no data exist for calculation of bond dispersions of other *cyclo*polymethylene groups bonded to silicon.

The bond refractions and bond dispersions obtained are collected in Tables 3 and 4.

The difference between the molar refraction (D-line) of a *cyclo*pentamethylenesilane and that of the corresponding *cyclo*tetramethylenesilane is about 4.50 ml. This value is only about 0.15 ml below the normal refraction of the methylene group the latter being 4.64 ml. (*cf.* Ref.[2], Table 40). The difference between the molar refraction (D-line) of *cyclo*hexamethylenedimethylsilane (No. 9) and that of *cyclo*pentamethylenedimethylsilane (No. 1) is 5.45 ml, a value which seems to be abnormally high. As mentioned previously the author has therefore not attempted to calculate the bond refraction of the *cyclo*hexamethylene group from the molar refraction of compound No. 9.

* Previously [2] the bond dispersion of a group X bonded to silicon was written Δ (Si—X). Here, however, the notation d (Si—X) is preferred.

Table 3. Bond refractions.

Bond refraction	C	D	F	g
r [$Si(CH_2)_5$]	26.77	26.90	27.22	27.47
r [$Si(CH_2)_4$]	—	22.42	—	—
r [$Si(CH_2)_3$]	—	18.06	—	—

Table 4. Bond dispersions.

Bond dispersion	F-C	g-C
d [$Si(CH_2)_5$]	0.459	0.712

The ring refraction in the *cyclo*trimethylene-, *cyclo*tetramethylene-, and *cyclo*pentamethylenesilanes can be estimated in the following way. A *cyclo*trimethylenesilane ring is formed from a methylethylsilane by the breaking of an α C—H and a β C—H bond and the formation of an α—β C—C bond. In the same way a *cyclo*tetramethylenesilane ring is formed from a diethylsilane by the breaking of two β C—H bonds and the formation of a β—β C—C bond. A similar procedure applied to an ethyl-*n*-propylsilane furnishes a *cyclo*pentamethylenesilane ring. In this case a β C—H and a γ C—H bond are broken and a β—γ C—C bond formed. The refractometric modifications involved may be expressed

$$r_D[Si(CH_2)_3] = r_D(Si—CH_3) + r_D(Si—C_2H_5) - 2r_D(C—H) + r_D(C—C) + R_1 \quad (4)$$

$$r_D[Si(CH_2)_4] = 2r_D(Si—C_2H_5) - 2r_D(C—H) + r_D(C—C) + R_2 \quad (5)$$

$$r_D[Si(CH_2)_5] = r_D(Si—C_2H_5) + r_D(Si—C_3H_7\text{-}n) - 2r_D(C—H) + r_D(C—C) + R_3 \quad (6)$$

where R_1, R_2 and R_3 are the ring refractions (D-line). Substitution of the appropriate values in eqs. 4, 5 and 6 gives $R_1 = 0.51$ ml, $R_2 = 0.36$ ml and $R_3 = 0.11$ ml. If the correct values of the C—H and C—C bond refractions were known the exact values of the ring refractions would be obtained. There is reason to assume that the values used in the calculation above for the C—H and C—C bond refractions are somewhat different from the real ones *. The error in the ring refractions caused from this, however, is not believed to exceed 0.10 ml. Thus it appears that a ring refraction is present in the *cyclo*trimethylenesilane and *cyclo*tetramethylenesilane rings while the ring refraction in the *cyclo*pentamethylenesilane ring seems to be negligible.

This is in agreement with the result of an inspection of Stuart molecular models of the three rings. While strain-free models may be built of the *cyclo*pentamethylenesilane ring this is not the case with the two other rings.

* The values of Denbigh [6] r_D(C—C) = 1.25 and r_D(C—H) = 1.69 were used.

It is of interest to note that Eisenlohr [7] in his refractometric investigations of *cyclo*butanes, *cyclo*pentanes and *cyclo*hexanes found the two latter types to be without ring refraction while the ring refraction of the *cyclo*butane ring was 0.46 ml. In a recent investigation Jeffery and Vogel [8] found the ring refraction of the latter ring to be 0.32 ml, a value which in all probability is more reliable than that of Eisenlohr. The greater strain in the silicon containing rings as compared with the corresponding carbon rings is evident from the calculations above. The considerable strain in the *cyclo*trimethylenesilane ring might explain the unusual reactivity of *cyclo*trimethylenedimethylsilane towards concentrated sulfuric acid and 1 N potassium hydroxide, reactions which proceed with ring opening.[4]

Use of the bond values. The bond refractions and bond dispersions in Tables 3 and 4 may be used for the calculation of molar refractions and molar dispersions of *cyclo*polymethylenedialkylsilanes and *cyclo*polymethylenedialkoxysilanes. In the former case this is simply made by addition of the bond values of the *cyclo*polymethylene group and those of the alkyl groups. In the latter case eqs. 2 and 3 given in this paper are used. The bond values of the alkyl and alkoxy groups and the K_r- and K_d-values are taken from Ref.[3].

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Water Solubility of Ethyl Cellulose

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A series of ethyl celluloses have been prepared from spruce sulphite pulp. The mercerization was carried out with soda lye of 24 to 40 % concentration; subsequent etherification with ethyl chloride. The degree of substitution varies from 0.9 to 1.35. The solubility in water shows a pronounced maximum at the degree of substitution 1.2.

The solubility properties of ethyl cellulose change with increasing ethoxyl content. At first it is soluble only in alkaline solutions, then in water and lastly only in organic solvents. As is well known this is the same sequence as for methyl cellulose but, owing to the stronger hydrophobic character of the ethyl group, the range of water-solubility is rather small.

Scattered information about the degree of ethylation which gives solubility in water is obtainable from literature. Berl and Schupp [1] etherified alkali cellulose from linters with diethyl sulphate and obtained water soluble products within the DS range 0.7—1.5 (DS = degree of substitution). The products must have been rather degraded as in one case the viscosity of a 3 % solution was only 57 cp. Bock [2] made the reaction in homogeneous medium. Cellulose was dissolved in quarternary ammonium bases and etherified with diethyl sulphate. A product with DS 0.6 was soluble in water whereas DS 1.2 gave insolubility.

Other figures are given by Traill [3] (DS 1.2), Lorand [4] (DS 0.8—1.3), Mahoney and Purves [5] (DS 0.6 and 1.2, resp.), Sönnerskog [6] (DS 1.2—1.4), but in all these cases very little information is given about raw material, etherification and method for measuring the solubility.

Some information is also found in patent literature [7-9]. It is claimed that ethyl celluloses with DS between 1.0 and 1.5 are soluble in cold water.

The widely differing figures and the lack of information in most cases about experimental conditions seem to justify a description of a series of experiments which were made as part of a broader research programme.

It is expected that the solubility of an ethyl cellulose will depend not only upon the DS but also upon the method of manufacture (homogeneity of etherification) and the molecular weight. It is also necessary to specify how the solubility was measured.

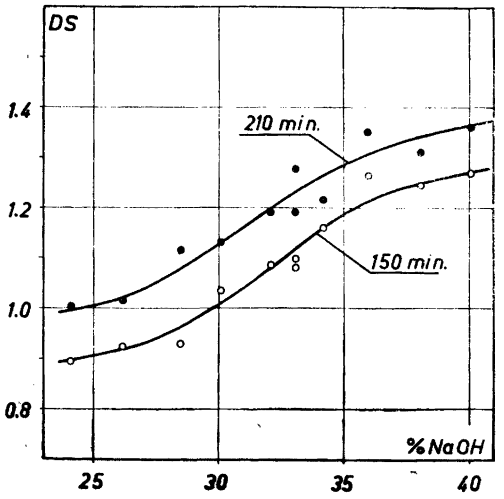

Fig. 1. Degree of substitution (DS) of ethyl cellulose as function of strength of mercerizing lye. Time of reaction 150 min. and 210 min. respectively.

Standard viscose sulphite pulp from spruce was mercerized and etherified in autoclaves with ethyl chloride. The resulting DS depends upon the time and temperature of the reaction and the strength of the mercerizing lye. The temperature cannot be varied very much, because below 90° C the reaction is very slow and above 110°—120° C the ether formed is rapidly degraded. In order to get a reasonably uniform substitution, the time of reaction should not be too short and the reaction is preferably broken off shortly before all the alkali has been used up. If it is allowed to proceed further, a hydrolytic breakdown of the chain molecules will start. In practice the main variable will therefore be the strength of the lye.

Raw material: Sulphite cellulose from spruce with alpha content 89.7 % [10] and viscosity 26 cp (1 % solution in cuprammonium [11]).

Cellulose sheets were mercerised 45 minutes at a temperature of 21°—24° C, squeezed to a press factor 2.5—2.6 (2.2 for the two lowest lye concentrations), and then shredded and aged for 35—60 minutes at room temperature. The autoclave (1 lit. capacity, stainless steel) was charged with alkali cellulose corresponding to 40 g air-dry cellulose and with 200 ml ethyl chloride. Another part of the alkali cellulose was regenerated with dilute acetic acid and the cuprammonium viscosity determined. The autoclave was rotated in an oil bath, the temperature of which was increased to 100° C over a period of 1.5 hours and then kept at 102°—104° C for 150 or 210 min. The raw product was suspended in boiling water, neutralized with acetic acid, sucked off on a Büchner funnel, and dried.

The ethoxyl analysis was made by the method of Vieböck and Schwappach [12] with modifications of Samsel and Mc Hard [13]. The ethoxyl percentage given is based on bone-dry and salt-free cellulose ether.

Viscosities of 2 % aqueous solutions of the cellulose ethers were measured with a Höppler viscometer, precision model HV 303. Transmission measurements on the same solutions were made by a method previously used in the study of ethyl-hydroxyethyl cellulose [14]. Lumetron photoelectric colorimeter, model 402—E, filter M 550, light path in cuvette 2 cm, displacement of cuvette 12 cm.

The cuprammonium viscosities were determined by the Swedish standard method CCA 16, the values recalculated to CCA 13 (TAPPI) [11].

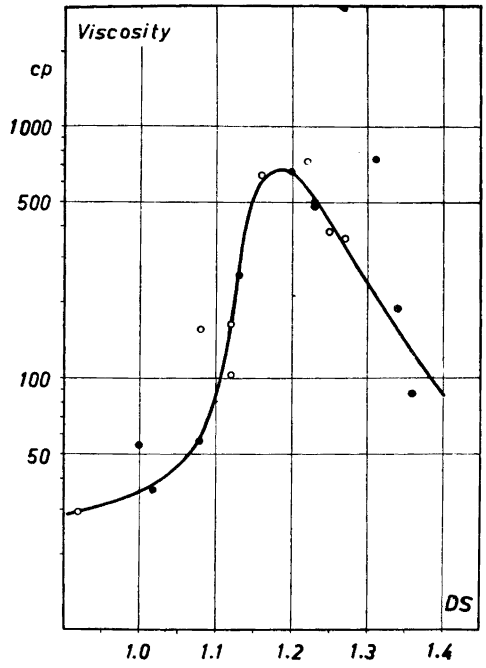

Fig. 2. Viscosity of 2 % solutions of ethyl cellulose which have been frozen, as function of degree of substitution. Temp. 20° C. For explanation of points, see Fig. 1.

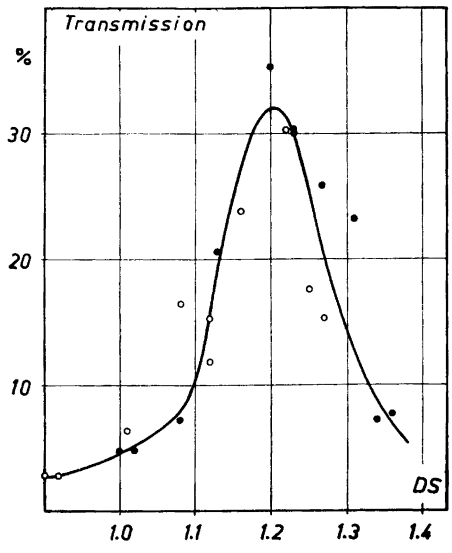

Fig. 3. Transmission of same solutions as in Fig. 2.

The degree of substitution as function of the lye concentration is shown in Fig. 1. Quadruple analyses were made in some cases, duplicate analyses in the other; standard deviation 0.02. The overall variation in DS is not very large and a closer study of the diagram shows that a considerable part of the spreading of the points may be due to the analytical errors. For that reason smoothed DS values were used as abscissae in the diagrams to follow.

The viscosity and turbidity of 2 % solutions of cellulose ether in water were measured and used as estimate of the solubility, a loosely defined concept for substances of this kind. A unique property of water-soluble cellulose ethers is that the viscosity of their solutions often increases irreversibly by cooling and subsequent warming back to the original temperature [14]. It is therefore necessary not only to state the temperature of measurement but also the minimum temperature to which the solution has ever been brought. With the exception of Sönnerskog [6] no information of this kind has been given in the literature references quoted.

The ethyl celluloses turned out to be typical cases of products showing irreversible viscosity increase after cooling. At 20° C they all gave a suspension of swollen fibers rather than a solution. Viscosity and turbidity measurements could not be carried out. Decrease of the temperature to 15° C did not improve matters but, after freezing, viscous solutions were obtained (Figs. 2 and 3). There is a sharp viscosity and transmission maximum around DS 1.2

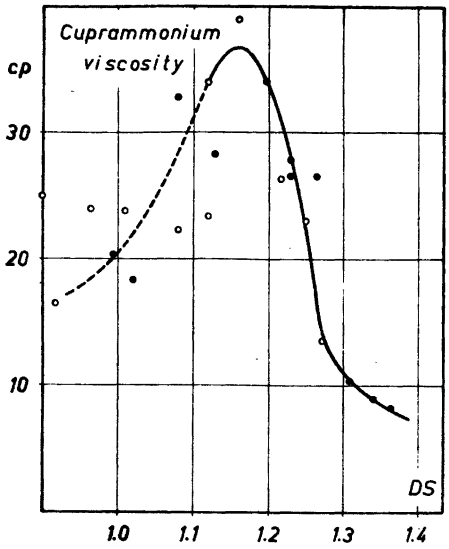

Fig. 4. Cuprammonium viscosity (1 % solution, TAPPI method) of ethyl cellulose as function of degree of substitution. For explanation of points, see Fig. 1.

at which point the steric influence of the substituted groups in keeping the cellulose chains apart and the hydrophobic character of the ethyl groups balance each other.

Determinations of the molecular weight were not carried out, but some information is gained from cuprammonium viscosity measurements on cellulose regenerated from the alkali cellulose immediately before the start of the etherification, and on the cellulose ethers. The first series of values decreased a little from 21 to 17 cp with increasing soda lye concentration; the second series is shown diagrammatically in Fig. 4. Even though the values spread very much at low DS the existence of a maximum seems well established. It is also remarkable that most viscosities are higher than those found for the regenerated cellulose. The same thing has also been repeatedly observed in the case of ethyl-hydroxyethyl cellulose [15]. No direct measure of the degradation which occurs during the etherification is available but the values in Fig. 4 indicate that it must be moderate.

The high values and the existence of a maximum can both be explained by the assumption that the chains get stiffer with increasing degree of substitution and that the hydration decreases because of the hydrophobic character of the ethoxyl groups. Highly etherified ethyl cellulose is not soluble in cuprammonium [1]. The result is a viscosity maximum at the same DS that gave a solubility maximum in water.

No doubt the solubility range will become broader with decreasing average chain length and this may explain some of the low DS values reported in literature. A more efficient way of increasing the water solubility is co-etherification with ethylene oxide [16,14].

Acknowledgement. Most of the experiments described in this paper were carried out by Mr G. Tjernström. The author expresses his sincere thanks for his careful work.

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Studies of Terpene Mixtures

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Two-component mixtures of the terpenes, Δ^3-carene, *d*-limonene, and α- and β-pinene purified to index homogeneity have been analysed using a micro sorption method. The sorptograms — n_D^{25} plotted against the 5 μl fraction number — are given for these six mixtures. The sensitivity of the analytical method has been studied for the different types of sorptograms.

As an appendix the sorptograms of some commercial samples of terpenes are given. No direct conclusion regarding the general qualities of the samples must be drawn from these sorptograms.

The determination of the purity of terpene hydrocarbons is of fundamental importance for the study of the reactions of individual terpenes. The earlier lack of methods available for testing the homogeneity of the liquid terpene hydrocarbons, used as starting materials for syntheses and isomerizations, has greatly hampered the interpretation of the results obtained. Simonsen [1] gives several examples of this kind in his comprehensive survey of the terpenes.

Collecting the results from a large series of experiments performed at Naval Stores Research Division, USA, O'Connor and Goldblatt [2] have shown it possible to determine the content of isomeric terpenes in some mixtures by infrared spectroscopy, and, from ultraviolet absorption curves, to classify the terpenes into groups. Widmark has, as described in earlier communications [3, 4], purified Δ^3-carene, *d*-limonene, and α- and β-pinene and studied the results obtained with an analytical micro sorption method devised by Blohm[5]. The present authors[6] have investigated the use of this method on oxidized Δ^3-carene.

In this investigation a series of mixtures of the four terpenes purified to index homogeneity (see above) has been analysed by the micro sorption method[5]: One drop (40.0 μl) was displaced with ethanol at pressure (1 atm) through a 25.0 cm long, narrow (i ø 1.4 mm) column filled with active silica gel and 5.0 μl fractions were collected, refractive indices of which were determined. With most of the mixtures very good readings were obtained and small amounts of the other terpenes were detected.

All the samples analysed here have been shown to be free from oxidation products, as even a slight oxidation can give rise to considerable changes in

the sorptograms [6]. A freshly oxidized sample, however, can be restored to its original stage by methanol extraction [3, 4].

The sequence, *e.g.* falling affinity to the gel, of these terpenes on sorption of 50 % mixtures is found to be *d*-limonene, Δ^3-carene, β-pinene, and α-pinene and the sorptograms — n_D^{25} plotted against the 5 μl fraction number — obtained after sorption of the mixtures have different shapes, *cf.* Figs. 1—6. After sorption of a two-component mixture containing one terpene as a minor constituent, the variation can be found in the beginning or at the end of the sorptogram. The method is usually most sensitive for those mixtures giving sorptograms with peaks at the ends. Thus 0.2 % Δ^3-carene can be detected in pure α-pinene. The sorptograms having an upward or downward slope in the beginning, *e.g.* α-pinene in Δ^3-carene, are less sensitive for interpretation purposes, but a few percent can be detected satisfactorily. Small amounts of Δ^3-carene and *d*-limonene in β-pinene give sorptograms with downward slopes at the end and usually less than 5 % cannot be detected in this case. When about 25 % or less β-pinene is present in Δ^3-carene there is a very small separating effect and straight sorptograms can be obtained; *cf.* Hirschler and Amon [7] who elucidate these phenomena in their investigation of separation of petroleum products on gel and carbon. The values of the refractive indices of Δ^3-carene and *d*-limonene are too close to allow good readings of the sorptograms and the sorption fractions have to be investigated with other micro identification methods. These results demonstrate clearly the danger in accepting the sorptogram as the sole criterion of purity.

Isomerization of the terpenes investigated caused by the passage through the active gel has not been observed yet, but the possible occurrence of these reactions will be further studied. The degree of activation is, however, of great importance, especially in the sorption of β-pinene mixtures, where only freshly activated gel will give reproducible results.

As an appendix to this paper, sorptograms of commercial samples of terpenes are given. For several reasons it is impossible to transfer directly the results obtained with the mixtures of the index homogeneous terpenes over to the commercial samples. The usefulness of the analytical method can as yet be considered as unknown for oxygen-containing terpenes and the method has already been found unsuitable for viscous liquids, or mixtures containing a solid compound. Furthermore, reservations have to be made when interpreting the quality of samples of the different manufactures from the sorptograms given here, as it is not known how our samples have been handled at the dealers. Nevertheless some of the examples given demonstrate, when used under controlled conditions, how the quality of terpene samples can be investigated using the sorption method.

EXPERIMENTAL PART

A micro sorption apparatus (column 250 × i ø 1.4 mm), fully conforming to Blohm's [5] instructions, was used. The silica gel (Davison, 922—08—226 through 200 mesh) was activated 2 hours at 140° (15 mm Hg). On reading the refractive indices, a fresh paper, ~ 5 × 5 mm, was used for each determination. The refractometer, Bellingham & Stanley No. 402330, was calibrated against a standard plate n_D 1.5009. The thermostat was

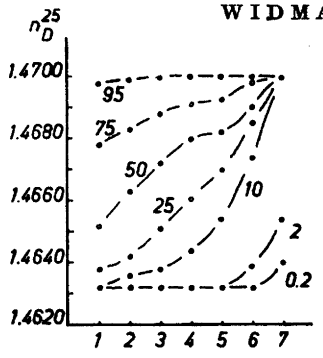

Fig. 1. Sorptograms of Δ^3-carene — α-pinene mixtures. Percent Δ^3-carene is given.

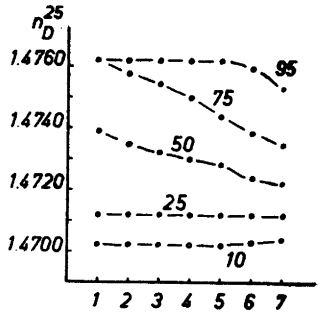

Fig. 2. Sorptograms of Δ^3-carene — β-pinene mixtures. Percent β-pinene is given.

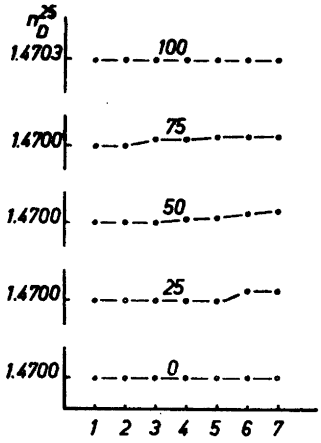

Fig. 3. Sorptograms of Δ^3-carene — d-limonene mixtures. Percent d-limonene is given.

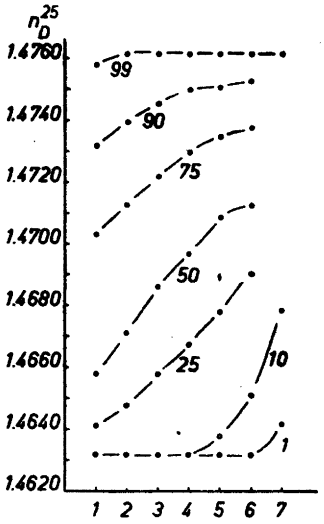

Fig. 4. Sorptograms of α-pinene — β-pinene mixtures. Percent β-pinene is given.

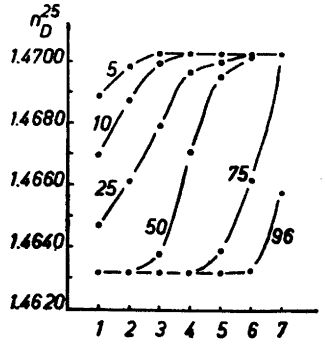

Fig. 5. Sorptograms of α-pinene — d-limonene mixtures. Percent α-pinene is given.

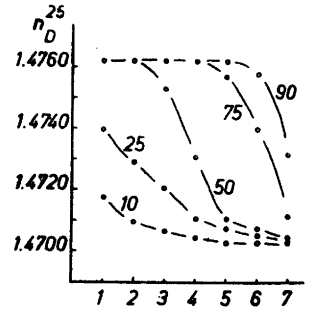

Fig. 6. Sorptograms of d-limonene — β-pinene mixtures. Percent β-pinene is given.

controlled to 25° ± 0.02°. The reproducibility of the sorptograms was found to be ± 0.0001 at the flat parts of the curves and max. ± 0.0005 at the steepest parts.

The terpenes, Δ^3-carene, *d*-limonene, and α- and β-pinene were purified to index homogeneity, *cf.* Widmark [3,4]. The mixtures were prepared by weighing in lots of about 0.5 g and stored in closed, nitrogen-filled tubes in an ice box.

The commercial samples were kept under nitrogen in tightly stoppered bottles, at room temperature and in a dark room, after arrival in the laboratory. No information on time of storing etc. was obtained from the dealers.

Sorptograms of the terpene mixtures are given in Figs. 1—6.

SORPTOGRAMS OF COMMERCIAL SAMPLES

The n_D^{25} values of the sorption are given with the fraction number as index. The value of the unsorbed sample is given US as index.

Fenchene. (Fluka, pract.) 1.4858_{US} 1.4714_{1-2} 1.4770_3 1.4940_4 1.4998_5 1.4874_6.

Dipentene. (Fluka) 1.4567_1 1.4682_2 1.4728_3 1.4745_4 1.4768_5 1.4804_6 1.4788_7; (Hopkins and Williams) 1.4748_1 1.4801_2 1.4809_3 1.4812_{4-6} 1.4817_7; (Schuchardt) 1.4742_{US} 1.4677_1 1.4672_2 1.4692_3 1.4728_4 1.4758_5 1.4807_6 1.4699_7.

d-Limonene (Eastman-Kodak) 1.4703_{US}, $_{1-6}$ 1.4710_7; (E-K tech.) $1,4703_{1-5}$ 1.4706_6 1.4717_7; (Hopkins and Williams) 1.4713_{US} 1.4654_1 1.4703_2 1.4709_3 1.4710_4 1.4715_5 1.4720_6 1.4747_7; (Fluka) 1.4770_{US} 1.4696_1 1.4715_2 1.4742_3 1.4765_4 1.4798_5 1.4763_6; (Schuchardt) 1.4720_{US} 1.4703_1 1.4722_2 1.4728_{3-5} 1.4739_6.

Phellandrene. (Fluka, tech.) 1.4783_{US} 1.4762_1 1.4753_2 1.4758_3 1.4797_4 1.4854_5 1.4832_6

α-Pinene. (Light) 1.4632_{1-5} 1.4637_6 1.4640_7.

p-Cymene. (Eastman-Kodak) 1.4853_1 1.4884_2 1.4883_{3-7}.

Carvone. (Schuchardt) 1.4955_{US} 1.4959_{1-3} 1.4960_{4-6} 1.4708_7.

Citral. (Fluka) 1.4867_{US} 1.4878_{1-3} 1.4865_4 1.4860_{5-6} 1.4690_7.

Citronellal. (Fluka) 1.4501_{US} 1.4518_1 1.4503_2 1.4496_3 1.4506_4 1.4554_5 1.4562_6.

Citronellol. (Fluka) 1.4535_{US} 1.4553_1 1.4532_{2-3} 1.4536_4 1.4541_5 1.4468_6.

Fenchone. (Fluka, purum) 1.4596_{US} 1.4595_1 1.4597_{2-6}.

Geraniol. (Fluka) 1.4692_{US} 1.4743_1 1.4703_2 1.4699_{3-5} 1.4682_6.

α-Ionene. (Fluka) 1.4967_{US} 1.4968_1 1.4965_2 1.4943_3 1.4882_4 1.4818_5 1.4718_6.

β-Ionone. (Fluka) 1.5126_{US} 1.5138_1 1.5131_2 1.5123_3 1.5128_4 1.5032_5 1.4900_6.

Linalool. (Fluka) 1.4597_{US} 1.4596_1 1.4597_2 1.4598_{3-5} 1.4575_6.

Menthone. (Fluka) 1.4492_{US} 1.4478_1 1.4482_{2-4} 1.4492_5 1.4504_6 1.4447_7.

Pseudoionone. (Fluka) 1.5263_{US} 1.5267_1 1.5273_{2-4} 1.5267_5 1.5197_6.

α-Terpineol. (Fluka) 1.4803_{US}, $_{1-4}$ 1.4760_5 1.4700_6.

Acknowledgements. The authors are indebted to Mrs. B. Holm for helpful assistance in the analytical work. The investigation has been supported by the *Swedish Medical Council.*

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The Reaction Mechanism of Yeast Alcohol Dehydrogenase (ADH), Studied by Overall Reaction Velocities

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Previous authors have investigated the kinetics of the yeast ADH system at pH 7.9. This pH was unsuitable for distinguishing between different possible reaction mechanisms, because the maximum overall reaction velocities (V_f and V_r) in both directions happened to be similar. We have now found that V_f and V_r are greatly different at pH 7.15 and 6.0, and new kinetic data are presented. These data are consistent with the postulate that coenzyme and substrate form a ternary complex with the enzyme, in which a rate limiting intramolecular reaction takes place. The affinity of DPN for the enzyme was found to increase with increasing concentration of ethanol, and *vice versa*, whereas the affinity of DPNH decreased with increasing concentration of acetaldehyde and *vice versa*.

The reaction mechanism of Alberty postulating oxidation-reduction of the enzyme protein itself with the formation of enzyme-substrate, or enzyme-coenzyme intermediates was found unlikely from our experimental data. Our data exclude the reaction mechanism probably occurring in the liver ADH system, which required that a ternary complex, if formed, must be shortlived in comparison with the enzyme-coenzyme complexes.

An enzymatic method for preparing reduced diphosphopyridine nucleotide has been described.

Equations relating Michaelis constants, maximum velocities and the equilibrium constant have been developed [1,2,3] in kinetic approaches to the selection of possible mechanisms of enzyme action. The relationship between these constants presents a means of testing the possibility of a particular mechanism.

Such an approach to the mode of action of yeast alcohol dehydrogenase has been carried out by Negelein and Wulff [4] and by Hayes and Velick [5,6]. However, their data were obtained at pH 7.9 which did not allow clear distinction between different possible reaction mechanisms. This is evident from

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the fact that the relationship between the equations as developed by Alberty [2], is determined by the ratio V_f/V_r*, and this ratio was less than 2 at that pH.

In an effort to obtain a greater V_f/V_r ratio and thereby to distinguish more definitely between the different alternatives, we determined the overall velocity constants at pH 7.15 and 6.0. It was found that V_f/V_r was 8.1 and 10, respectively, at these pH values. These conditions therefore were much more favorable for distinguishing between various possible mechanisms.

MATERIALS AND METHODS

Crystalline yeast alcohol dehydrogenase (yeast ADH)

A commercial preparation from C. F. Behringer & Soehne, G.m.b.H., Mannheim, was used. The maximum turnover number of this preparation with acetaldehyde and DPNH was found to be 30 000 min^{-1} at pH 7.15, and 23 000 min^{-1} at pH 6.0 (23°). At pH 7.9 Negelein and Wulff [4] found 15 600 min^{-1} for their preparation. The experiments were carried out in the presence of M/100 versene, since the enzyme is readily poisoned by heavy metals. However, yeast ADH has recently been shown to contain zinc [7], which might be essential for the activity and removed by versene. We have therefore investigated the velocity at pH 7.15 in the presence and absence of 0.001 M versene. No effect of versene was observed under these conditions.

DPN AND DPNH

(Oxidized and reduced diphosphopyridine nucleotide).

DPN was prepared according to the method of Neilands and Åkeson [8], and this preparation was reduced enzymatically to obtain DPNH.

DPNH was prepared by modification of existing methods, the main difference being that boiling of the solution to inactivate the enzyme was avoided. To 25 ml of 0.1 M sodium carbonate/bicarbonate buffer pH 9.6 was added 0.4 ml ethanol, 300 μg yeast ADH, and 1 ml of DPN solution (20 mg/ml in H_2O). During the reaction period, three additional 300 μg portions of ADH, four 1 ml portions of DPN solution, and four portions of 0.4 ml ethanol were added. When about 90 % of the DPN was reduced, the solution was placed under vacuum for some 5 minutes, to remove acetaldehyde, the pH was adjusted to 10.0 and a last addition of enzyme finally made. 80 ml ethanol were added and the solution kept at $-10°$ C for 10 minutes. The precipitate containing the enzyme was removed by centrifugation and discarded. DPNH was precipitated by adding 320 ml ethanol + 400 ml ether at $-10°$. The precipitate flocculated in 5—10 minutes, was collected by centrifugation, washed with ether and dried. The yield was approximately 70 % of the theoretical. There remained 2—4 % unreduced DPN. A solution containing 60 mg DPNH per ml was only moderately yellow.

The oxidation of DPNH or reduction of DPN was followed by fluorescence measurements as described previously [9]. For concentrations of DPNH above 16 μM, a sensitive recording spectrophotometer was used. This apparatus was constructed at this institute and will be described elsewhere. The use of these instruments allowed more accurate determinations of the kinetic constants than can be obtained in an ordinary Beckman spectrophotometer.

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* V_f = maximum velocity for forward reaction.
V_r = » » » reverse »

All the kinetic constants presented in this paper have been determined twice and found to check within ± 10 % with the exception of those obtained at low concentration of the second reactant where the deviations were somewhat higher.

Alberty [2] has introduced a new type of kinetic constant, called K_{AB}, which may be calculated by several different methods from kinetic data at concentrations of A and B, insufficient to saturate the enzyme (for instance, A = DPN, B = ethanol in our case).

K_{AB} is a function of ordinary and "apparent" Michaelis constants:

$$K_{AB} = K'_A K_B + [B_0](K'_A - K_A) = K_A K'_B + [A_0](K'_B - K_B) \quad (1)$$

where K_A and K_B = ordinary Michaelis constants; $[A_0]$ and $[B_0]$ = concentration of A or B at $t = 0$; and K'_A and K'_B = apparent Michaelis constants for A and B, respectively. The apparent Michaelis constant is determined in the same way as is the ordinary K_m, except that the concentration of the reactant which is kept constant (the second reactant) is made insufficient to saturate the enzyme, preferentially below K_m. We have determined K_{AB} in both ways indicated in the equation above and presented the average value in Table 1, column I, and in Table 2.

Our sensitive fluorimetric and spectrophotometric methods made it possible to determine with a fair degree of accuracy the initial velocities even in such cases where both A and B were low and the changes occurring in the concentration of DPNH therefore small and decreasing rapidly within a few seconds.

RESULTS AND DISCUSSION

Linear relationships have been obtained between reciprocal initial velocities and reciprocal concentrations of substrate and coenzyme over large concentration ranges, and no deviation from straight lines were observed at pH 6.0. However, at pH 7.15, deviation towards higher activity took place when the concentration of DPN and alcohol were very high (See Fig. 1). This occurred above 100 mM alcohol in the presence of 800 μM DPN, but was not observed in the presence of 38 μM DPN and 280 mM alcohol, nor in the presence of 6.2 mM alcohol and 800 μM DPN. There are of course several possible explanations for this activation. For example, the catalytic reaction of the ADH·DPN·alcohol complex may be increased due to changes in the charge distribution on the surface of the enzyme at alcohol concentrations high enough to influence the dielectric constant. The activation is not observed at low DPN concentrations because other steps may be rate limiting. In this connection it may be recalled that liver ADH was inhibited by concentrations of alcohol higher than 5 mM. Under the conditions studied, the dissociation of the ADH·DPNH complex was the rate-limiting reaction, and this complex may have been stabilized by the high alcohol concentrations due to changes in the dielectric properties of the medium. This is in agreement with the interpretation of the effect of salts on this reaction.

In the following calculations, all data relate only to the linear parts of the plots.

Table 1. Kinetic data from Table 2 inserted into four formulae representing different reaction sequences [2].

Buffer: phosphate, μ = 0.1 + versene, M/1000	I Ternary complex formed, relatively slow intramolecular transformation of the complex. General mech. of Alberty [2] $K_{eq}^{*} = \frac{V_f}{V_r} \cdot A^{**} \times 10^{11}$	II Special case of I. Affinity of ADH for DPN(H) not influenced by substrate, or *vice versa* $K_{eq} = \frac{V_f}{V_r} \cdot B^{***} \times 10^{11}$	III A group in the enzyme is oxidized/reduced by substrate (or coenzyme) with the formation of enzyme-substrate intermediates $K_{eq} = \left(\frac{V_f}{V_r}\right)^2 \cdot B \times 10^{11}$	IV Relative stable ADH · DPN(H) complexes, shortlived ternary complex formed $K_{eq} = \left(\frac{V_f}{V_r}\right)^3 \cdot B \times 10^{11}$
pH 7.15	1.1	17.9	2.2	0.27
pH 6.0	0.81	4.5	0.45	0.045

* $K_{eq} = 0.9 \times 10^{-11}$ over the whole pH range.

** $A = \frac{K_{ald} \cdot DPNH}{K_{alc} \cdot DPN}$; For the definition of these constants, see Alberty [2].

*** $B = \frac{K_{DPNH} \cdot K_{ald}}{K_{DPN} \cdot K_{alcohol}}$

Table 2. Michaelis constants and maximum reaction velocities for yeast alcohol dehydrogenase (23°). Buffer: phosphate, μ = 0.1 + versene, M/1000.

Concentration of second reactant		Kinetic Constants		
pH 7.15	pH 6.0		pH 7.15	pH 6.0
μM	μM		μM	μM
185	185	K'_m, DPNH	14	12
5000	5000	K'_m, DPNH	38	24
13.5	13.2	K'_m, aldehyde	250	140
150	80	K'_m, aldehyde	550	300
0.62×10^4	5.5×10^4	K'_m, DPN	200	167
84×10^4	84×10^4	K'_m, DPN	57	160
38	77	K'_m, ethanol	50000	96000
800	800	K'_m, ethanol	18000	100000
		$K_{DPNH} \cdot ald$ *	5500	1300
		$K_{DPN} \cdot alc$	4.2×10^6	16×10^6
		V_f, sec^{-1} **	61	38
		V_r, sec^{-1}	491	380
		V_f/V_r	0.124	0.100

* For definition of these constants, see Alberty [2]. The constants can be calculated in two ways, and average values have been used (see eq. (1)).

** V_f is maximum velocity of ethanol oxidation.
V_r " " " " aldehyde reduction.

Mol.wt. of enzyme is 150 000[5,6].

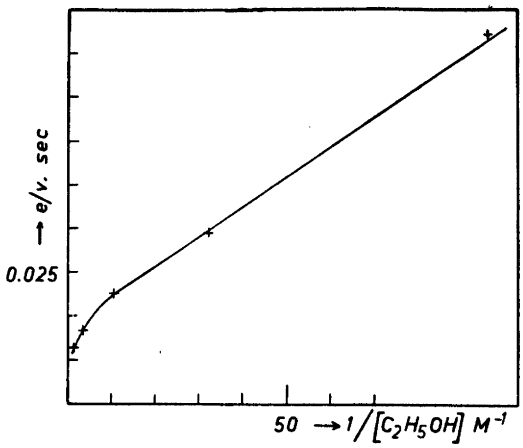

Fig. 1. Lineweaver-Burk plot of yeast alcohol dehydrogenase with varied alcohol concentration. Conc. of DPN: 770 μM. Buffer: phosphate, μ = 0.1 + M/1000 versene. pH = 7.15, 23° C.

It is apparent from Table 1 that case I agrees well with the experimental data. This equation was developed [2] by the steady state treatment of a reaction between enzyme and two substrates (one of the substrates may be coenzyme) to form a ternary complex, in which an intramolecular reaction occurs to form another ternary complex. The velocity of this reaction is assumed to be slow compared with the rates of formation and breakdown of binary and ternary complexes. From this relationship it is not possible to decide whether two or more intermediary complexes are formed, or whether the substrate and the coenzyme combine and dissociate in a certain order. However, the investigations of Hayes and Velick [5] have shown that binary complexes of the composition ADH·$(DPN)_4$ and ADH·$(DPNH)_4$ can be formed in the absence of substrate. Whether binary complexes of the type ADH·alcohol or ADH·acetaldehyde can be formed is not known. It has been reported that some acetaldehyde is nonspecifically attached to yeast ADH [4]. Liver ADH contains bound ethanol in non dialyzable form [10].

Case II represents the general mechanism described above with the additional assumption that the affinity of the coenzyme for the enzyme is uninfluenced by the concentration of substrate, and *vice versa*. Negelein and Wulff [4] and Hayes and Velick [5,6] in kinetic studies of yeast ADH at pH 7.9 found the equation presented in column II to agree well with their experimental data. However, it is clear from our data that yeast ADH does not follow this reaction mechanism at pH 7.1 and 6.0. First, the relationship between kinetic constants does not give the correct equilibrium constant (see Table 1). Second, as shown in Table 2, the apparent Michaelis constant, K_m, of DPN and alcohol, as well as DPNH and acetaldehyde were in most cases affected by changes in the concentration of the second reactant. K_m for alcohol and DPN decreased with increasing concentrations of the second reactant at pH 7.1, whereas at pH 6.0 there was no effect. K_m for acetaldehyde and DPNH (both at pH 7.1 and 6.0) increased with increasing concentration of the second reactant.

Case III presumes an oxidation-reduction of the enzyme itself with the formation of enzyme-substrate intermediates. The involvment of thiol groups of DPN-linked enzymes in hydrogen transfer has been suggested by several authors [11,12], but strong arguments have been presented against such a mechanism for yeast ADH [13,14]. The results obtained with deuterium-labeled substrate exclude all mechanisms of yeast alcohol dehydrogenase which allow hydrogen exchange with the medium. Such an exchange would probably have taken place if thiol groups were directly involved in the hydrogen transfer. Case III is therefore improbable from previous experiments. Our own data may not be accurate enough to eliminate this reaction scheme. In the unlikely case that 10 % error in the determination of each of the different kinetic constants added up in the same direction, the error in K could give a deviation from 0.9 to 0.44 or from 0.9 to 2.2 (See Table 1).

In case IV the assumption has been made that the ternary complex is extremely short-lived and dissociates into products much more rapidly than do the binary complexes (ADH·DPN and ADH·DPNH).

In some recent experiments [9] we have confirmed that liver ADH appears to follow Case IV. Considering the great difference in the properties of yeast and liver ADH [5,6] it is not surprising that the two enzymes could catalyze the same reaction in different manners. A further difference was revealed by the reaction velocities in the presence of sodium chloride. This salt had no effect on V_f and V_r for the yeast enzyme, whereas V_f was increased and V_r was decreased with the liver enzyme [9]. At relative low concentration of the second reactant, yeast ADH was competively inhibited by 0.15 M NaCl using alcohol, DPN and DPNH as variable reactants. The effect of formate [9], however, was qualitatively the same with both enzymes: strong inhibition was observed except in the presence of high alcohol concentrations.

Thanks are due to Dr. William Rutter for valuable discussions and to Miss Ann Louise Swanson for skilful technical assistance.

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Electron Diffraction Studies on the Molecular Structures of Pyridine and Furan

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Electron diffraction studies have been carried out on the structures of pyridine and furan. The results have been compared with those obtained from the microwave spectra [1,2]. The obtained structure parameters found are summarized in Tables 1 and 2.

Microwave studies have led to very accurate determinations of the molecular parameters of pyridine [1], and furan [2]. An independent electron diffraction study using the sector method [3] promised to provide valuable informations as to the molecular structures of the compounds. It would also determine whether the two methods, which are quite different in principle, lead to the same results. The electron diffraction studies were carried out on the same samples used in the microwave work.

Pyridine. Fig. 1 shows the intensity curve of pyridine obtained after subtraction of the background. Various different radial distribution curves have been calculated both from the undamped intensity curve and from intensity curves multiplied by various damping factors. Fig. 2 shows the radial distribution curve calculated using a damping factor of $e^{-0.0009s^2}$.

Each of the peaks in the radial distribution curve is composed of contributions from two or more different distances. For example, the main peak at approximately 1.38 Å is built up of contributions from the C-C bonds and the C-N bonds; the corresponding distances are too close together to be separated. In this comparison of the microwave and the electron diffraction results, therefore, average values are involved.

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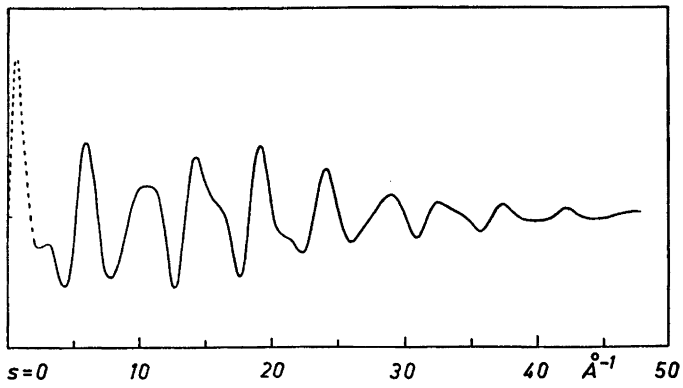

Fig. 1. Intensity curve for pyridine.

Table 1. Mean distances in pyridine obtained from the microwave spectra and from the electron diffraction studies.

Distances	H_2-C_2 H_3-C_3 H_4-C_4	$N-C_2$ C_2-C_3 C_3-C_4	H_2-N H_2-C_3 H_3-C_2 H_3-C_4 H_4-C_3	$N-C_3$ C_2-C_4 C_2-C_6 C_3-C_5	$N-C_4$ C_2-C_5	H_2-C_6 H_2-C_4 H_3-N H_3-C_5 H_4-C_2	H_2-C_5 H_3-C_6 H_4-N
Microwave model V	1.081	1.377	2.137	2.384	2.753	3.358	3.821
Electron dif. values	1.078	1.377	2.141	2.384	2.748	3.356	3.802

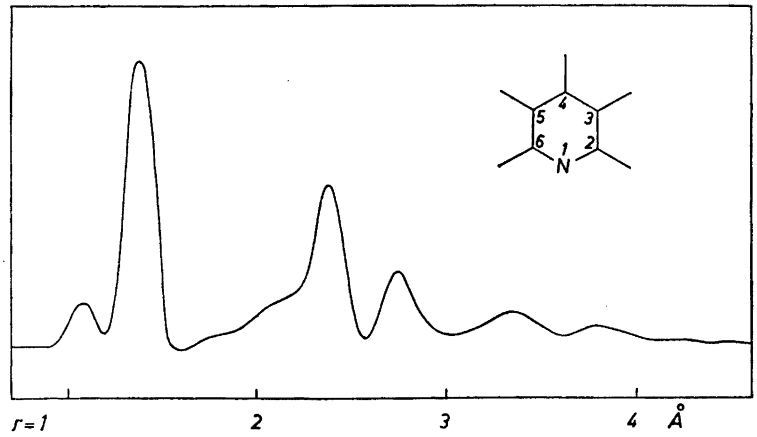

Fig. 2. Radial distribution curve for pyridine.

In Table 1 the best electron diffraction data have been listed, together with the data calculated from the best microwave model[1]. All of the distances contributing to the mean distances are listed in the first row. The numbering of the atoms can be seen from Fig. 2. The correspondence between the values obtained from the two different methods is extraordinarily good; it is, as a matter of fact, somewhat better than one would expect in view of the probable errors of the methods. Because of the many, indeed very small, sources of uncertainty it is difficult to make a definite statement concerning the probable error of the various distances obtained from the electron diffraction studies. However, the following qualitative discussion may be helpful. The peaks at 1.078, 2.384 and 2.748 Å seem all to be highly reproducible. The various ways of approaching the problem seem to give very nearly the same values for the average distances corresponding to these peaks. The study of the intensity curve and of the radial distribution curves based upon various damping factors give essentially the same values, *i.e.* only a variation of a few thousandths of an Ångstrom. The maximum of the peak at 2.141 Å is not determined with this high accuracy, however. The peak is so close to its neighbour at 2.384 Å that a different procedure has to be applied. The position of the maximum is reproduced with a maximum error of 0.012 Å. The peaks at 3.356 and 3.802 Å are small and might, therefore, not be expected to be determined very accurately. On the other hand they are well resolved, and the deviation between the microwave and the electron diffraction values is negligible in the first case and only about 0.5 % in the second case.

The peak at 1.377 Å seems on the first inspection to lead to some difficulties. Although this peak is the predominating one there is a definite increase in the position of its maximum when the damping factor increases. The undamped intensity curve leads to a value of 1.371 Å, and the value increases to a limit of 1.377 Å when the damping factor is increased. This effect is very reproducible, but so small as to raise questions as to experimental errors, such as magnetic fields in the apparatus, for example. The effect on the most pronounced peak in other compounds has, therefore, been studied. In addition to pyridine the effect was observed only in the case of furan. The explanation is probably the following: Every distance contributes to the intensity curve by a damped sine-like function. The damping is dependent upon the variation in the distances because of molecular vibrations, so that the "stiffer" the distance the less the damping. Now, the various bonds contributing to the peak under discussion have different force constants. If the plausible assumption is made that the shorter bonds are the stiffer ones, these bonds should dominate the outer range of the intensity curve. Accordingly the radial distribution curves based upon undamped intensity curves should have the main peak shifted toward small r-values. The artificial damping has a tendency to equalize the contribution from the various bonds. This is the reason for believing the average distance calculated from the damped intensity curve to be the best value.

Furan. Fig. 3 is the intensity curve and Fig. 4 the radial distribution curve for furan. Various radial distribution curves were also calculated for furan by the same procedure as was used for pyridine. The results are summarized in Table 2, where the best electron diffraction data are compared with the two

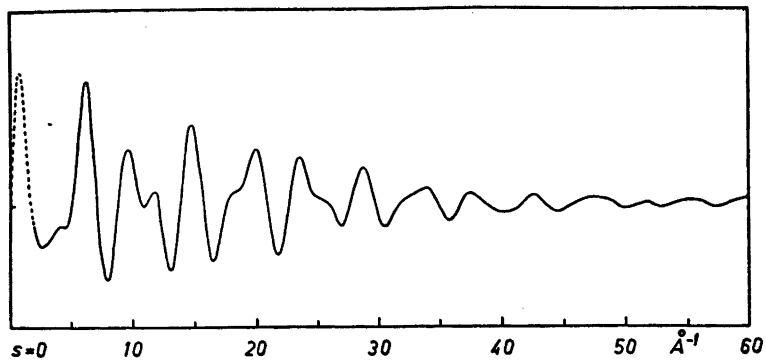

Fig. 3. Intensity curve for furan.

best models obtained from the microwave studies by Bak, Hansen and Rastrup-Andersen [2]. From the electron diffraction work it is impossible to distinguish between the two microwave models. Unfortunately, the average distance

Table 2. Mean distances in furan obtained from the microwave spectra and from the electron diffraction studies.

Distances	H_2-C_2 H_3-C_3	$O-C_2$ C_2-C_3 C_3-C_4	H_2-O H_2-C_3 H_3-C_2 H_3-C_4	$O-C_3$ C_2-C_4 C_2-C_5	H_2-C_4 H_2-C_5 H_3-C_5 H_3-O
Microwave model III	1.075	1.378	2.186	2.229	3.260
» IV	1.075	1.378	2.185	2.228	3.261
Electron diffraction	1.075	1.377		2.230	3.272

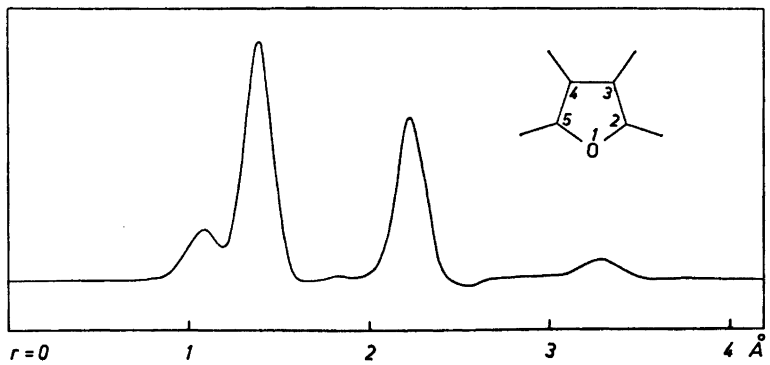

Fig. 4. Radial distribution curve for furan.

from hydrogen to carbon, and hydrogen to oxygen through one angle cannot be determined, as it is too close to the r-value of the peak at 2.230 Å. As mentioned above, in this case as well the position of the main peak is dependent upon the artificial damping factor. The peak occurs in the undamped case at 1.370 Å.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 1311—1316

*iso*Thiocyanates XIV. 5-Methylthiopentyl *iso*Thiocyanate, a New Mustard Oil Present in Nature as a Glucoside (Glucoberteroin)

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Seeds of the crucifer *Berteroa incana* (L.) DC. appear to contain three *iso*thiocyanate glucosides (A, B and C) as estimated by paper chromatography. On enzymic hydrolysis, one of these, *viz.* (C), for which the name *glucoberteroin* is suggested, affords a volatile *iso*thiocyanate, $C_7H_{13}NS_2$, different from all previously known natural mustard oils. Its thiourea-derivative has been prepared and demonstrated to be identical with an authentic specimen of N-(5-methylthiopentyl)-thiourea (II), synthesized *via* the previously unknown 5-methylthiopentyl *iso*thiocyanate (I) in an unambiguous series of reactions. Evidence is presented of the identity of the second glucoside (A) as the sulphoxide (VIII), corresponding to glucoberteroin, whereas the chemical nature of the third glucoside (B) remains to be elucidated. Glucoberteroin seems to be rather widely distributed in nature.

In a previous paper of this series [1] seeds of the crucifer *Berteroa incana* (L.) DC. were mentioned as the source of a steam-volatile *iso*thiocyanate, meriting further investigation.

Recently, Schultz and Gmelin [2,3] reported the appearance of three glucoside spots in paper chromatograms of methanolic extracts of seeds of *B. incana*. The spot of lowest R_F-value, belonging to their "Gruppe II", was considered to represent a glucoside of unknown nature, whereas the other two spots were attributed to sinalbin and glucotropaeolin, respectively. The same general pattern was obtained on repetition here (Fig. 1), although the interpretation needed thorough revision. This became evident when the seed material was submitted to enzymic hydrolysis and a characteristic radish-like smell developed, due to the liberation of an *iso*thiocyanate, which could be removed by steam-distillation and transformed into a thiourea upon treatment with ammonia. Paper chromatography in chloroform demonstrated the presence of only one thiourea, possessing an R_{Ph}-value [4] of 1.13. This finding immediately excluded the presence of benzylthiourea (R_{Ph} 0.92) and conse-

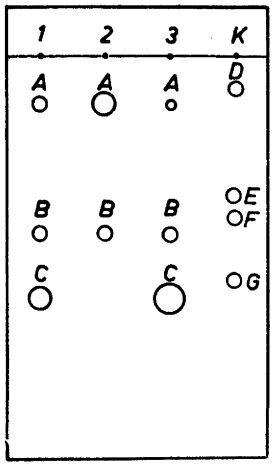


Fig. 1. Descending paper chromatogram in n-*butanol : acetic acid : water (4 : 1 : 3) of : 1. a freshly prepared extract of seeds of* Berteroa incana *(L.) DC.; 2. a two-week old extract of the same seeds; 3. the aged extract after short treatment with a hot solution of* $Na_2S_2O_4$*; K. a control solution. A : sulphoxide of glucoberteroin (VIII); B : unknown glucoside; C : glucoberteroin; D : glucoiberin* [3]*; E : sinigrin; F : sinalbin and G : glucotropaeolin.*

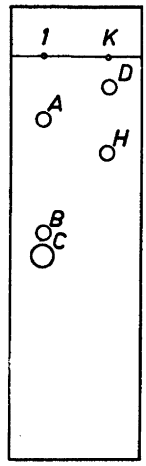


Fig. 2. Descending paper chromatogram in pyridine : amyl alcohol : water (35 : 35 : 30) of 1. a freshly prepared extract of seeds of B. incana*; K. a control solution. A, B, C and D : the same as in Fig. 1.; H : glucocheirolin, applied as an extract of seeds of* Cheiranthus cheiri *L.*

quently also of glucotropaeolin. Furthermore, paperchromatographic data proved the volatile *iso*thiocyanate to be different from all previously known, natural members of this class. From a larger seed sample, cultivated during the summer of 1954 in the Botanical Garden here, it was possible to isolate the thiourea of the new *iso*thiocyanate in pure, crystalline form. It was devoid of optical activity and analyses indicated the composition $C_7H_{16}N_2S_2$. Consequently, it could be inferred that the new *iso*thiocyanate, $C_7H_{13}NS_2$, possessed a sulphur-containing side-chain.

Recently we demonstrated by isolation the presence in nature of 3-methylthiopropyl [5] and 4-methylthiobutyl [6] *iso*thiocyanate, both genuinely occurring in seed materials as glucosides, *viz.* glucoibervirin and glucoerucin, respectively. In analogy we wish to suggest the name *glucoberteroin* for the parent glucoside of *B. incana* (L.) DC., giving rise to the $C_7H_{13}NS_2$-mustard oil on enzymic hydrolysis. It appeared reasonable to regard the latter as a methylthio-derivative also, not least on account of its radish-like smell being so similar to that of the other methylthioalkyl *iso*thiocyanates. The rather limited amounts of thiourea on hand did not permit a direct structure proof by degradation. Hence, it was assumed as a working hypothesis that the C_7-*iso*thiocyanate could be a further member of the straight-chain methylthio-series, *viz.* the

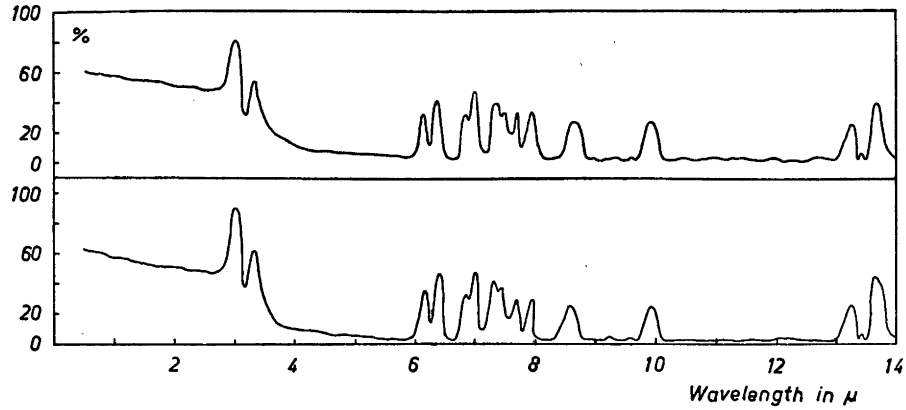


Fig. 3. Infrared spectra determined on a Beckman IR-2 instrument in potassium bromide discs. Upper curve : thiourea, derived from the volatile isothiocyanate *of* Berteroa incana *seeds. Lower curve : synthetic N-(5-methylthiopentyl)-thiourea (II). Ordinate: Absorption in per cent.*

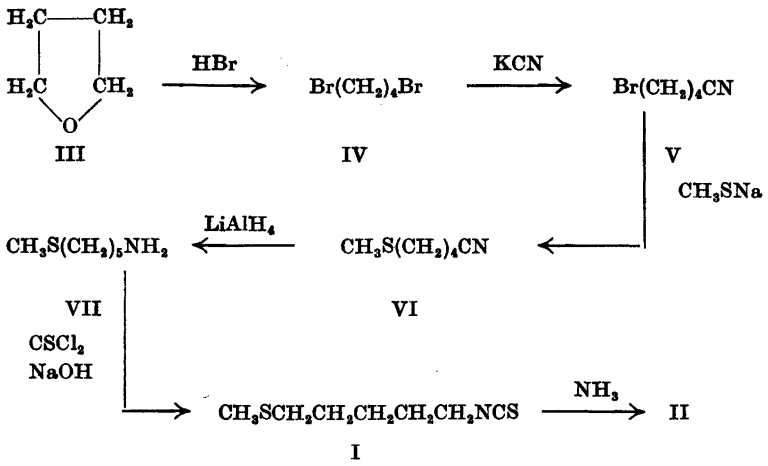
previously unknown 5-methylthiopentyl *iso*thiocyanate (I); a synthetic approach was undertaken to test this supposition.

$CH_3SCH_2CH_2CH_2CH_2CH_2NCS$ I

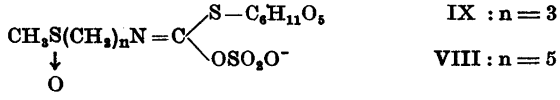
$CH_3SCH_2CH_2CH_2CH_2CH_2NHCSNH_2$ II

Tetrahydrofuran (III) was transformed into 1,4-dibromobutane (IV) [7] and the latter in turn into 5-bromovaleronitrile (V) with potassium cyanide [8]. Reaction of (V) with sodium methylmercaptide afforded in quantitative yield 5-methylthiovaleronitrile (VI) which was smoothly reduced with lithium aluminium hydride to 5-methylthiopentylamine (VII), characterized as its derivative with 2,4-dinitrochlorobenzene. The amine appears to have been used by Karrer *et al.*[9] for further syntheses with no record, however, of experimental or physical data. Thiocarbonyl chloride and alkali [10] reacted with (VII) to the high-boiling 5-methylthiopentyl *iso*thiocyanate (I), possessing a characteristic radish-like smell, undiscernible from that of enzymically hydrolyzed extracts of seeds of *B. incana*. The *iso*thiocyanate was finally transformed into the thiourea (II) by reaction with ammonia. The identity of the latter with the thiourea derived from the natural *iso*thiocyanate, established by paper chromatography, mixed melting point determination and comparison of infrared spectra (Fig. 3), proved beyond doubt the presence in nature of 5-methylthiopentyl *iso*thiocyanate (I).

It was incidentally noted that storage of seed extracts of *B. incana* was accompanied by the disappearance of glucoberteroin in the paper chromatograms (Fig. 1). This observation provided the clue to identification of the glucoside (A). A suspicion, that the latter might be the sulphoxide (VIII), corresponding to glucoberteroin, was confirmed by paper chromatography in pyridine : amyl alcohol : water (Fig. 2), a solvent system which has proved very useful for the purpose of distinguishing between glucosides, possessing



sulphoxide- and sulphone-groupings in their side-chains. An R_F-value lower than that of glucocheirolin, containing the short methylsulphonylpropyl-side chain, excluded the identity of the Berteroa-glucoside (A) as a sulphone. On the other hand, the presence of a rather long sulphoxide-containing chain in the molecule was suggested through its location on the paper, definitely above that of glucoiberin [3] (IX) (Fig. 2).



Further support for the above interpretation was provided in a more direct way. When an aged methanolic extract, devoid of glucoberteroin, was subjected to reduction with sodium dithionite and chromatographed, a strong spot of glucoberteroin appeared at the expense of the sulphoxide-derivative (VIII) (Fig. 1). This result leaves little doubt as to the occurrence of (VIII) even in freshly prepared seed extracts of *B. incana*. Although genuinely present in plant material, (VIII) is most likely to consider as a secondary product, formed by oxidation *in vivo* of glucoberteroin.

The third glucoside (Fig. 1, B), appearing in the chromatograms at about the site of sinalbin, is being further studied at the present. No colour is produced on its exposure to diazotized sulphanilic acid, a reaction previously shown to be diagnostic for the presence of sinalbin [3]. On enzymic hydrolysis the glucoside affords a non-volatile *iso*thiocyanate.

The occurrence of glucoberteroin is by no means confined to *B. incana*. So far, we have proved it to be present also in seeds of *Lunaria rediviva* L. as well as in numerous species of *Alyssum*. An account of the distribution of glucosides within the latter genus will be presented in a forthcoming communication.

EXPERIMENTAL

All melting points are uncorrected and determined in capillary tubes in a water-bath. Paper chromatography was performed on Whatman paper No. 1 by the descending technique in a constant temperature room.

Isolation of the volatile isothiocyanate as thiourea. Seeds of *Berteroa incana* (L.) DC. (27.2 g) were crushed and defatted with a mixture of ligroin and ethanol by our usual procedure [1]. The dried seed powder (22.1 g) was suspended in water (150 ml), to which was added a myrosinase-solution (1.5 ml), and the mixture was left at room temperature for 16 hours. The radish-smelling *iso*thiocyanate was removed by steam distillation and collected in conc. ammonia (100 ml). Next day the solution was concentrated to dryness *in vacuo*, leaving a colourless oil which soon crystallized (121 mg). The product was recrystallized from 30 % ethanol and separated as colourless, flat prisms (78,5 mg). Another recrystallization, this time from ethyl acetate and pentane, afforded an analytical specimen (58.5 mg) as nacreous platelets, m.p. 67°. The ultraviolet spectra were determined in both water and 96 % ethanol and represented typical thiourea-absorption ($\lambda_{max}^{H_2O}$ 237 mμ (ε 12 500); λ_{max}^{EtOH} 243 mμ (ε 13 700)). A solution of the thiourea (13.9 mg) in EtOH (1.02 ml) did not give any observable optical rotation. (Found : C 43.55; H 8.17; N 14.38; S 33.06. Calc. for $C_7H_{16}N_2S_2$: C 43.71; H 8.39; N 14.57; S 33.35).

Paper chromatography of the thiourea in chloroform as usual [4] showed it to be homogenous with an R_{Ph}-value of 1.13, easily discernible from β-phenylethylthiourea (R_{Ph} 1.06), possessing the highest R_{Ph}-value of all thioureas derived from previously recognized, natural *iso*thiocyanates.

Syntheses

1,4-Dibromobutane (IV). This compound was prepared from tetrahydrofuran (III) and hydrogen bromide, essentially as described by Wilson [7]; b.p. 77.5° at 11 mm. Yield 77 %.

5-Bromovaleronitrile (V). The nitrile was prepared from a large excess of the dibromide (783 g) and potassium cyanide (47.1 g), as reported by Leonard and Wildman [8]. We found the reaction to be complete after 5 hours heating, where previously 24 hours were prescribed. B.p. 114—116° at 12 mm. Yield 46.4 g.

5-Methylthiovaleronitrile (VI). Methyl mercaptan (16 g) was slowly added to a solution of sodium (7.0 g) in dry methanol (175 ml). To this solution was added in one portion 5-bromovaleronitrile (46.4 g) dissolved in methanol (100 ml). The solution was refluxed for 7 hours and the methanol removed by distillation. Water (50 ml) was added to dissolve the salt and the solution repeatedly extracted with ether (100 ml). The extract was dried, the ether removed by distillation and the residue distilled *in vacuo* as a colourless liquid with a characteristic smell. Yield 33.7 g (91 %); b.p. 112—114° at 8 mm. (Found : C 55.70; H 8.57; N 10.42. Calc. for $C_6H_{11}NS$: C 55.76; H 8.58; N 10.84).

5-Methylthiopentylamine (VII). Finely powdered lithium aluminium hydride (11.4 g) was dissolved in dry ether (250 ml) and the solution placed in a three-necked flask, provided with magnetic stirring, a separatory funnel, reflux condenser and a nitrogen inlet. In a slow stream of nitrogen, the nitrile (VI) (25.8 g), dissolved in ether (40 ml), was dropwise added over 30 minutes to the well-stirred reaction mixture. This was finally refluxed for 30 minutes in order to complete the reduction, and then cooled in an ice-bath. From the funnel were carefully added water (13 ml), 15 % sodium hydroxide (13 ml) and water again (45 ml). The precipitate was removed on a sintered glass filter and thoroughly washed with fresh portions of ether. The pooled ether solutions were dried over pellets of KOH, the ether slowly removed by means of a short Vigreux-column and the resulting amine distilled *in vacuo*. The colourless liquid distilled at 88° and 8 mm; n_D^{25} 1.4852. (Found : C 53.75; H 11.23; N 10.38. Calc. for $C_6H_{15}NS$: C 54.10; H 11.34; N 10.51). The yield was 20.3 g (76 %).

2,4-Dinitro-N-(5'-methylthiopentyl)-aniline. For the purpose of characterization a derivative was prepared from the above amine and 2,4-dinitrochlorobenzene in the usual way. Beautiful yellow needles were obtained upon recrystallization from methanol, m.p. 40°. (Found : C 47.96; H 5.83; N 13.98. Calc. for $C_{12}H_{17}O_4N_3S$: C 48.16; H 5.73; N 14.04).

5-Methylthiopentyl isothiocyanate (I). To a suspension of the amine (VII) (13.3 g) in water (75 ml) was slowly added a solution of thiocarbonyl chloride (12.7 g) in chloroform (100 ml), followed by 0.2 N NaOH (100 ml). The reaction proceeded rapidly under evolution of heat and after shaking for 15 minutes the dark-coloured layers were separated and the aqueous phase extracted with two additional portions of chloroform. The organic layer was dried and the solvent removed by distillation. The resulting *iso*thiocyanate distilled as a colourless oil at 155° and 10 mm; n_D^{25} 1.5422. Yield 12.3 g (70 %). The compound had a very penetrating smell of radish. (Found : C 48.25; H 7.51; N 8.09; S 36.48. Calc. for $C_7H_{13}NS_2$: C 47.97; H 7.48; N 7.99; S 36.58).

N-(5-Methylthiopentyl)-thiourea (II). A mixture of the *iso*thiocyanate (2.64 g), conc. ammonia (20 ml) and ethanol (20 ml) was left standing at room temperature for 48 hours. The now homogenous solution was evaporated to dryness *in vacuo* and the crystalline residue recrystallized from ethyl acetate and pentane (2.03 g, 70 %). An additional recrystallization from chloroform and pentane afforded the thiourea as colourless, rhombic plates of m.p. 67.5°, alone or in admixture with the above derivative, obtained from the volatile *iso*thiocyanate of *B. incana*. (Found : C 43.70; H 8.22; N 14.35. Calc. for $C_7H_{16}N_2S_2$: C 43.71; H 8.39; N 14.57). The infrared spectra of the two products were determined in potassium bromide discs and proved to be identical (Fig. 3). Paper chromatography likewise served to confirm the identity of the two thioureas.

Microanalyses were performed in this laboratory by Mr. P. Hansen. We wish to thank the *Botanical Garden of the University of Copenhagen* for the cultivation of *Berteroa incana* (L.) DC. on a larger scale.

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The Dialysis Technique in the Study of the Vitamins and Amino Acids Affecting Associations of Micro-organisms

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A dialysis cell with several compartments suitable for the study of the chemical nature of the associative, and especially symbiotic, inter-relationships among micro-organisms is described. Using this apparatus two symbiosis experiments with a five or ten compartment cell using three or seven different species of lactic acid bacteria, respectively, were made. The chemical factors affecting the close symbiosis between the different organisms were found to be certain amino acids and vitamins.

In connection with biosynthetic studies on growth factors using the symbiotic technique [1,2] a simple method was needed by which the direct contamination of different symbionts during growth could be prevented. In this paper a simple apparatus is described in which organisms are separated from each other by a dialysing membrane in a cell assembled with two or more compartments. In these experimental conditions the bacteria used can grow in associations composed of more than two species. Symbiosis experiments were made using this apparatus, in which from three to seven different species of lactic acid bacteria were able to grow together despite the fact that some growth factors essential for the symbionts were omitted from the basal medium. This has been found to be due to the fact that the growth factors produced by the bacteria in each compartment passed through the dialysing membrane to the adjoining compartment, so permitting symbiotic growth in the dialysis cell.

EXPERIMENTAL TECHNIQUE

Apparatus. The apparatus (Figs. 1 and 2) consists of two or more compartments made of Pyrex glass each furnished with a glass tube open at the top, which can be plugged with cotton wool. A detailed drawing of a dialysis cell with three compartments is shown in Fig. 1. A second and more complicated modification of the same apparatus with ten compartments is shown in Fig. 2. Each compartment has approximately 12—15 ml capacity (not including glass tube) except for the cross-shaped compartment (shown in Fig. 2) which has 50 ml capacity. A dialysis membrane (in Fig. 1 A) (Cellulose, No. 4465—A2, A. H. Thomas Co, U.S.A.) is placed between the compartments, the surfaces

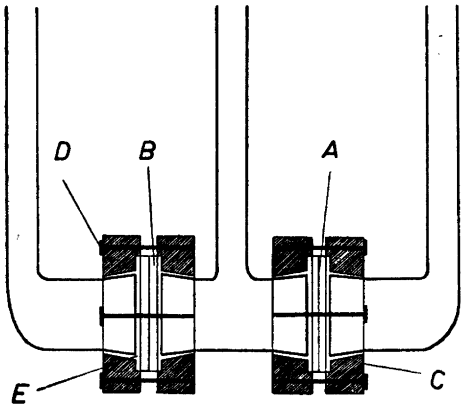

Fig. 1. Scheme showing a three-compartment dialysis cell (For description see the text).

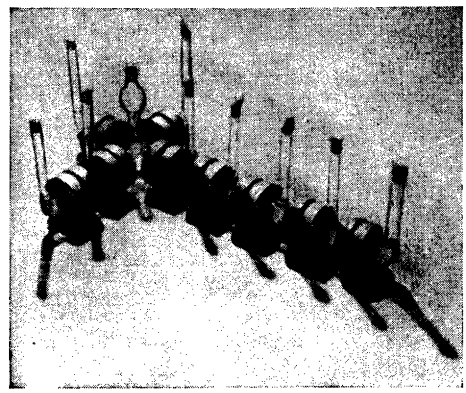

Fig. 2. Photograph showing a ten-compartment dialysis cell.

between the sections being ground smoothly, and held in place by rubber gaskets (B). The compartments are connected by aluminium hoops (C) equipped with set screws (D) and rubber packings (E). As the walls of the glass compartments and the rubber packings are slightly conical, the joints can be made water-tight by tightening the screws (D).

Cultures and Method. The organisms used were *Lactobacillus arabinosus* 17—5, *Lactobacillus casei*, *Lactobacillus fermenti*—36, *Leuconostoc mesenteroides* P—60, *Leuconostoc citrovorum* (ATCC 8081), *Streptococcus faecalis* R, *Streptococcus lactis* (ATCC 7963). The stock cultures were maintained by stab inoculation in the glucose-citrate-tryptone-yeast extract-agar as described previously [3], excluding one strain, *Str. lactis* (ATCC 7963), which was cultured in sterile skim milk. The inocula were prepared by transferring the organisms from the stab culture to 7 ml of glucose-citrate-tryptone-yeast extract medium, except for the last-mentioned strain, which was transferred to the inoculum medium as described by Anderson and Elliker[4]. After incubation for 16—18 hours at 37° C the cells were centrifuged out and washed with 0.9 % sterile saline. This process was repeated, the cells being washed 2—3 times. The cells were finally suspended in saline. One drop of barely visible suspension was used as inoculum for approximately 5 ml of the final medium.

The basal media of Henderson and Snell [5] and Anderson and Elliker [4] in slightly modified form as described previously [1,6] were used. The special omissions of some vitamins and amino acids from the basal media are noted in the text. The basal medium was diluted with an equal volume of water and placed in the dialysis cell and also in test tubes as a control (to test tubes in 5 ml portions) and autoclaved at 112° C for 5 minutes. After cooling, the dialysis cell and test tubes were inoculated aseptically and then incubated at 37° C. The growth response was followed turbidimetrically. The extent of growth was recorded as galvanometer readings of a Klett-Summerson photoelectric colorimeter with a 660 mμ filter.

RESULTS AND DISCUSSION

In the symbiosis experiments a chemically defined medium was prepared, omitting certain vitamins and amino acids which were essential for the growth of the lactic acid bacteria used. The separate compartments of the dialysis cell were then inoculated with the different strains of bacteria (and in addition each organism as a control separately into the test tube containing this incomplete medium). It should be emphasized that both inoculum cultures were washed thoroughly and that very little inoculum was used in order to avoid

Table 1. The symbiotic growth of Lb. arabinosus *17—5 (phenylalanine-requiring),* Str. faecalis *R (folic acid or folinic acid-requiring), and* Ln. mesenteroides *P—60 (phenylalanine and proline-requiring) in a five-compartment dialysis cell for 68 hours at 37° C.* (cf. *Fig. 3*). *Basal medium without phenylalanine, folic acid, folinic acid, and proline.*

Organism	Colorimeter reading		
	in dialysis cell		in test tube
	compartment		
Lb. arabinosus 17—5	2	232.0	23.0
	4	230.0	
Ln. mesenteroides P'60	1	192.0	14.0
	3	204.0	
Str. faecalis R	5	56.0	12.0
None			3.0

transference of the missing growth factors with the cells (apart from what the cells themselves contained) to the incomplete medium.

As can be seen from Table 1, *Str. faecalis* R, *Lb. arabinosus* 17—5, and *Ln. mesenteroides* P—60 could not grow alone in the test tubes, but were able to grow when inoculated into separate compartments of a cross-shaped dialysis cell despite the fact that three essential growth factors, phenylalanine, proline, and folic acid, were omitted from the basal medium (the basal medium of Henderson and Snell modified by the author was used in this experiment). Of these growth factors phenylalanine was required by *Lb. arabinosus* 17—5 and *Ln. mesenteroides* P—60, folic acid by *Str. faecalis* R, and proline by *Ln. mesenteroides* P—60. The growth of bacteria in the dialysis cell must be due to each organism releasing into the medium the growth factor required

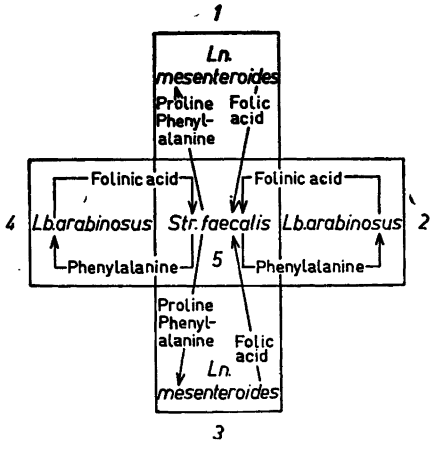

Fig. 3. The growth factors affecting the symbiotic growth of Lb. arabinosus *17—5,* Str. faecalis *R, and* Ln. mesenteroides *P—60 in a five-compartment dialysis cell.* (Cf. *the experiment described in Table 1*).

Table 2. The symbiotic growth of Lb. arabinosus *17—5 (phenylalanine-requiring),* Lb. fermenti—36 *(phenylalanine-requiring),* Lb. casei *(phenylalanine and folic acid or folinic acid-requiring),* Str. faecalis *R (folic acid or folinic acid-requiring),* Ln. mesenteroides *P—60 (phenylalanine-requiring),* Ln. citrovorum *(8081) (folinic acid-requiring), and* Str. lactis *(7963) in a ten-compartment dialysis cell for 69 hours at 37° C.* (Cf. *Fig. 4*). *Basal medium without phenylalanine, folic acid and folinic acid.*

Organism	Colorimeter reading		
	in dialysis cell		in test tube
	compartment		
Str. lactis 7963	1	39.0	48.5
	4	77.0	
Str. faecalis R	2	75.0	8.5
	8	39.0	
	10	48.0	
Lb. casei	3	96.0	9.0
Ln. citrovorum 8081	5	44.0	7.5
Lb. arabinosus 17—5	6	197.0	14.0
Lb. fermenti—36	7	78.0	4.0
Ln. mesenteroides P—60	9	44.0	7.5
None			2.0

by the strain of the adjoining compartment and to the passage of the factors through the dialysis membrane. Fig. 3 shows schematically in which compartments of the dialysis cell the different species of bacteria are situated and which are the essential growth factors produced by each organism. As can be seen, the central compartment of the dialysis cell was inoculated with *Str. faecalis* R. This organism was able to synthesize phenylalanine and proline and excrete these amino acids into the medium during growth. On the other hand, *Str. faecalis* R obtained the vitamin folic acid (pteroylglutamic acid) required by it from the adjoining compartments. This compound was produced by *Lb. arabinosus* 17—5 and *Ln. mesenteroides* P—60. On the basis of the earlier findings [1,6] it may be concluded that the folic acid produced by *Lb. arabinosus* 17—5 existed in its catalytically active form folinic acid (citrovorum factor, 5-formyl-5,6,7,8-tetrahydrofolic acid) a compound which possesses high folic acid activity for *Str. faecalis* R.

The second and more complicated symbiosis experiment is described in Table 2 and Fig. 4. This experiment was made with seven different species of lactic acid bacteria using the ten compartment dialysis cell illustrated in Fig. 2. The basal medium of Anderson and Elliker (modified by the author) was used, omitting the essential growth factors phenylalanine, folic acid, and folinic acid from the medium. Among the organisms used *Ln. citrovorum* 8081 required for growth folinic acid (citrovorum factor) and phenylalanine, *Lb. casei* folic acid (or folinic acid) and phenylalanine, *Str. faecalis* R folic acid (or folinic acid), *Lb. arabinosus* 17—5, *Lb. fermenti*—36, and *Ln. mesenteroides* P—60 phenylalanine. One strain, *Str. lactis* 7963, did not require these compounds. On the contrary, in an earlier work [6] it had already been shown that this

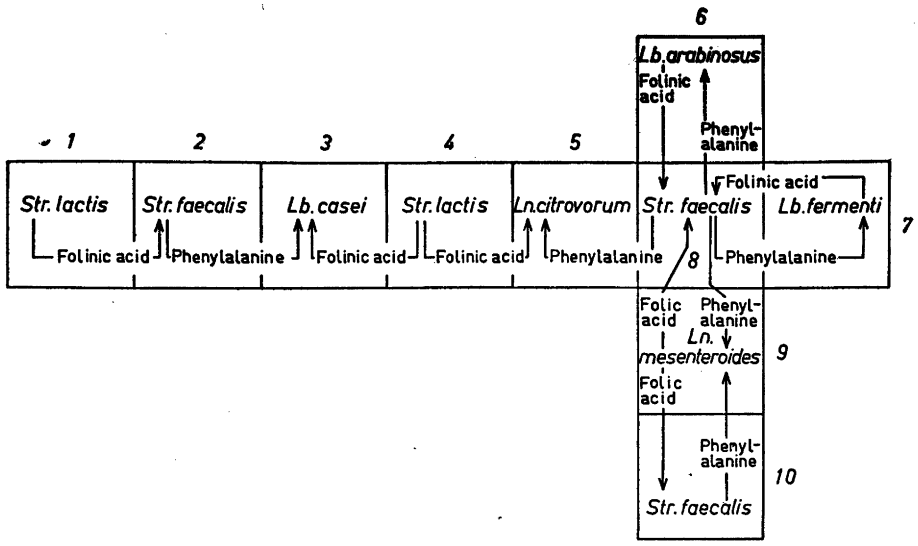

Fig. 4. The growth factors affecting the symbiotic growth of Lb. arabinosus *17—5*, Lb. fermenti *—36*, Lb. casei, Str. faecalis *R*, Str. lactis *(7963)*, Ln. mesenteroides *P—60, and* Ln. citrovorum *(8081) in a ten-compartment dialysis cell.* (Cf. *the experiment described in Table 2).*

organism was capable of producing folinic acid into the medium during growth and that phenylalanine was only a slightly stimulatory growth factor for it. As can be seen from Table 2 and Fig. 4, in the present experiment *Str. lactis* 7963 excreted folinic acid into the medium, so permitting the growth of *Ln. citrovorum* 8081, *Lb. casei*, and *Str. faecalis* R in the adjoining compartments. *Str. faecalis* R also obtained folic acid or folinic acid from the other strains, namely from *Lb. arabinosus* 17—5, *Lb. fermenti*—36, and *Ln. mesenteroides* P—60. These three last-mentioned organisms, on the other hand, benefited from *Str. faecalis* R, which was capable of producing phenylalanine into the medium.

In the symbiosis experiments described here, the growth of different species of bacteria in the dialysis cell must be due to that each organism during growth produced into the medium the growth factor (vitamin or amino acid) required by the strain of the adjoining compartment, these compounds then passing across the dialysing membrane. The results are in good agreement with those obtained in earlier symbiosis investigations [1] and are particularly interesting in view of the fact that in natural conditions micro-organisms live in mixed populations with complex inter-relationships. It seems likely that in nature also, in mixed microbial cultures, the production and excretion of growth factors such as vitamins and amino acids plays an important role in the formation of the associative relationships among micro-organisms.

In addition to symbiosis experiments with lactic acid bacteria the dialysis technique was also used in the study of the biosynthesis and the excretion of

vitamins and amino acids into the medium with the growing cells of certain other bacteria (*e. g. Escherichia coli*). In the course of this work (in progress) it has appeared that the dialysis cell described here is very useful in investigations of this kind. Evidently, the dialysis technique has also other possible applications in the investigation of the chemical nature of the associative relationships existing between micro-organisms, a field in which there are in general very few useful methods available.

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Methylated Taurines and Choline Sulphate in Red Algae

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Taurine, N-methyltaurine, di-N-methyltaurine and choline sulphate have been isolated from red algae. From these results a metabolic pathway from taurine *via* N-methylated taurines and choline sulphate to polysaccharide sulphate esters might be indicated.

During an investigation of the low-molecular weight carbohydrates of the red alga *Furcellaria fastigiata*, a substance which proved to be di-N-methyltaurine was isolated [1]. The crude product, free from carbohydrates, was difficult to purify by crystallisation, suggesting that it was contaminated by substances of similar structure.

By a similar procedure, taurine has now been isolated from *Ptilota pectinata* and *Porphyra umbilicalis*. This observation was followed by a more systematic search for similar compounds in red algae. The three N-methylated taurines were prepared and a method to separate and characterise them by paper chromatography was developed. Taurine, N-methyltaurine and di-N-methyltaurine could be detected with an indicator reagent, bromothymol blue, previously used for the detection of ammonium salts of fatty acids [2]. The spots, yellow on a blue background, faded rapidly and the two first substances were preferably detected with the ninhydrin reagent. The colour reaction of N-methyltaurine was not as sensitive as that of taurine and a higher temperature was required to produce the colour. Tri-N-methyltaurine could not be detected by these reagents. The first three substances could be separated in a number of solvent systems of which acetone-water (9 : 1) proved quite convenient. As expected, taurine had the lowest and di-N-methyltaurine the highest R_F-value.

The red alga *Gelidium cartilagineum*, the principal agar weed used on the American west coast, was especially rich in these substances, containing about 1.5 % of internal salts. Evidence for the presence of taurine, N-methyltaurine and di-N-methyltaurine was obtained by paper chromatography, and the last two were also isolated and proved to be identical with the corresponding authentic materials (mixed melting points). For the attempted isolation of tri-N-methyltaurine, a fraction of internal salts free from carbohydrates, was filtered through a column of Dowex 2 in the OH-state. Tri-N-methyltaurine, being an internal salt of a strong base and a strong acid, would not be expected

to be absorbed in contrast to the others, which are internal salts of a weak base and a strong acid. A substance was isolated which, most unexpectedly, was not tri-N-methyltaurine but was recognised as choline sulphate by its I.R. absorption spectrum. The mother liquors from the choline sulphate were treated with 5 N hydrochloric acid at 100° overnight, and the solution was deionised and concentrated. No tri-N-methyltaurine could be detected in the minute residue. The presence of small amounts of tri-N-methyltaurine is, however, not excluded by these experiments, as it might have accumulated in other fractions.

N-Methyltaurine and di-N-methyltaurine have not been found in Nature before, while taurine itself is known to occur in the muscular tissue of invertebrates and also in vertebrates as a constituent of the bile acids. Unfortunately a lot of diatoms grow on the alga investigated. Most of these were removed prior to the extraction but the complete exclusion of them was practically impossible. A small amount of mytilitol (0.002 %) was isolated from the extract, and this C-methylinositol, previously isolated from the edible mussel, *Mytilus edulis* [3], probably comes from invertebrates in the sample extracted. The percentage of the taurines, however, is so high that it seems to exclude the possibility that these compounds are derived from impurities in the plant material.

Choline sulphate has been isolated from an *Aspergillus* [4] and a *Penicillium* [5] mould and from lichens of the family *Roccella* [6]. The isolation of this substance from a red alga is of special interest, as red algae contain a high percentage of galactosan sulphate esters (agar), and it seems reasonable to put forward the hypothesis that the sulphate ester groups are transferred from choline to the polysaccharide by a transesterification. The simultaneous occurrence of choline sulphate and the taurines might indicate a biochemical relationship between these substances, *e. g.* that taurine is successively methylated to tri-N-methyltaurine, which is then, by some sort of oxidation, transferred into choline sulphate.

$$H_3\overset{+}{N}CH_2CH_2\overset{-}{S}O_3 \longrightarrow (CH_3)_3\overset{+}{N}CH_2CH_2\overset{-}{S}O_3 \overset{?}{\longrightarrow} (CH_3)_3\overset{+}{N}CH_2CH_2O\overset{-}{S}O_3$$

EXPERIMENTAL

(Melting points uncorrected)

Ptilota pectinata. The alga (130 g) was extracted with ether for 2 days and with methanol for 14 days. The methanol extract was concentrated to dryness, the residue treated with water, filtered and the aqueous phase deionised with IR 120 and IR4B and concentrated. The sirup (1.1 g) was dissolved in hot aqueous ethanol and on cooling crystals (170 mg) separated. By paper chromatography the presence of taurine and small amounts of N-methyltaurine was demonstrated, and by further crystallisation pure taurine, m.p. 320–325° (decomp.) was obtained (Found: C 19.1; H 5.60. Calc. for $C_2H_7O_3NS$: C 19.2; H 5.64.)

Porphyra umbilicalis. The isolation of an "internal salt" fraction from this alga has been reported in a previous communication [7]. It melted at 300–315° (decomp.) and gave a strong spot corresponding to taurine and a faint spot corresponding to N-methyltaurine.

Gelidium cartilagineum. The alga (370 g) was extracted and worked up as described above for *Ptilota pectinata.* The crystals obtained from the sirup were recrystallised four times from aqueous ethanol yielding a fraction, G_1 (2.1 g) which was free from carbo-

hydrates and melted at 270—280° (slight decomp.). The last three mother liquors were concentrated yielding another carbohydrate free fraction, G_3 (2.1 g). The remaining mother liquors were combined, yielding G_3 (14.0 g).

G_3 was fractionated on a carbon column using the gradient elution technics as previously described [1]. Floridoside (6.3 g), m.p. 127—128°, was isolated from some of the last fractions. The early fractions, containing internal salts, glycerol and cyclitols were concentrated and filtered through a column of Dowex 2 in the OH^- state and the column washed with water. The eluate, which had an odour of trimethylamine, contained the carbohydrates and when it was concentrated a small quantity of crystalline material (8 mg), m.p. 258—262°, separated. The substance was chromatographically indistinguishable from mytilitol and the m.p. was undepressed on admixture with this substance. The mother liquors were concentrated to a sirup (0.37 g) in which the presence of glycerol, mesoinositol and laminitol [8] was demonstrated by paper chromatography. Any choline sulphate or tri-N-methyltaurine present in G_3 should be accumulated in this fraction. The column was then eluted with 2 N acetic acid (100 ml) and the eluate concentrated yielding crystalline material (1.35 g) which, by chromatographic evidence, consisted of a mixture of taurine, N-methyltaurine and di-N-methyltaurine. Part of this fraction (200 mg) was fractionated on thick filter paper (Whatman 3 MM) using as solvent acetone-water (19 : 1). Chromatographically pure N-methyltaurine (60 mg) was isolated by this procedure. After two crystallisations from aqueous ethanol the substance (34 mg) melted at 240—242°, undepressed on admixture with an authentic specimen.

Pure di-N-methyltaurine (70 mg) melting at 297—300° (decomp.) after one crystallisation was also isolated. Taurine was present only in small quantities and was obtained in a small fraction (11 mg) consisting of about equal parts of taurine and N-methyltaurine.

Fractionation of G_2 on a carbon column was attempted but was not very successful. The chief constituent of the fraction was di-N-methyltaurine, which was the only substance isolated in a pure state.

G_1 (1.8 g) was dissolved in water and filtered through a column (50 ml) of Dowex 2 in the OH^- state and the column washed with water (300 ml). The aqueous solution was concentrated to dryness and the residue (0.54 g) recrystallised twice from aqueous ethanol. The crystals (290 mg) on heating decomposed at about 315° without melting while tri-N-methyltaurine melts with decomposition at about 335°. The behaviour on heating was the same as that of choline sulphate and the I.R. absorption spectra of the two substances were identical.

The Dowex 2 column was eluted with 2 N acetic acid (150 ml) yielding crystals (1.18 g). By crystallisation from aqueous ethanol, pure di-N-methyltaurine, m.p. 300—302° (decomp.) was obtained.

Synthesis of methylated taurines. Sodium 2-bromoethane-sulphonate (11 g) was dissolved in a strong (25—33 %) aqueous solution (700 ml) of the amine [NH_2CH_3, $NH(CH_3)_2$ and $N(CH_3)_3$, resp.], and kept at room temperature for 10—14 days. The solution was then concentrated to dryness under reduced pressure, dissolved in water, deionised by filtering through columns of IR 120 and IR 4B, concentrated and crystallised from aqueous ethanol. The method is essentially the same as previously used for these substances, improved, however, by the use of ion exchange resins to remove the salts.

N-Methyltaurine	Yield 76 %	M.p. 244—245°
Di-N-methyltaurine	Yield 68 %	M.p. 299—304° (decomp.)
Tri-N-methyltaurine	Yield 75 %	M.p. 335—340° (decomp.)

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The Action of Bromine on Podophyllotoxin and Picropodophyllin

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The preparation of a new monobromo-derivative of podophyllotoxin was reported in a recent short communication. The position of the bromine atom has now been established by oxydative degradation with permanganate. The isolation of 2-bromo-3,4,5-trimethoxybenzoic acid proves that the structure of "bromopodophyllotoxin" is 1-hydroxy-4(2-bromo-3,4,5-trimethoxyphenyl)-6,7-methylenedioxy-2-hydroxymethyl-1,2,3,4-tetrahydro-naphthoic acid-(3)lactone (Fig. 4).

Hydrogenation with Raney-nickel catalyst leads to picropodophyllin. Bromopodophyllotoxin forms a crystalline acetyl derivative.

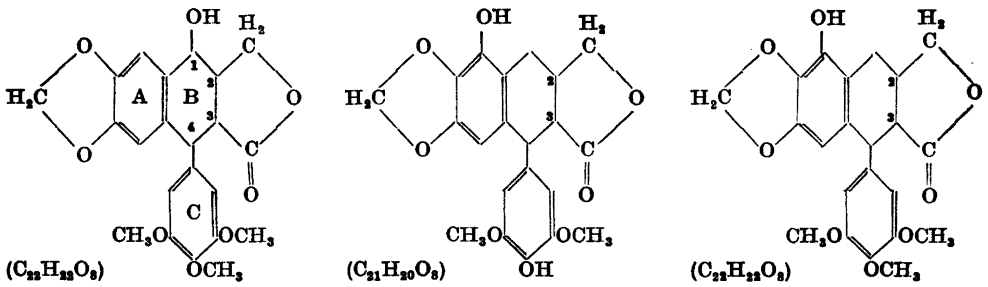
The epimeric compound, bromo-picropodophyllin, has been prepared both by base-catalyzed epimerization of bromopodophyllotoxin and by bromination of picropodophyllin.

By the action of bromine in a slight excess on a paste of powdered podophyllotoxin (Fig. 1, I) and glacial acetic acid Dunstan and Henry [1] obtained a bromo-derivative, which they described as colourless crystals melting above 250° C and containing 20.9 % Br. The empirical formula $C_{15}H_{14}O_6Br$ (calc. 21.6 % Br) was proposed, podophyllotoxin itself being erroneously ascribed the formula $C_{15}H_{14}O_6$.

By a similar procedure picropodophyllin (II) yielded a reaction product, which failed to crystallize, except from ethanol, and the dissolution in this medium was accompanied by liberation of free bromine.

Fractional precipitation with ether and light petroleum of a product, which had previously been recrystallized from ethanol, yielded a substance melting at 138° C and containing 18.85 % Br. The authors held the view that it was an isomeride of bromo-podophyllotoxin.

A different bromo-derivative of podophyllotoxin was obtained by Hartwell and Schrecker [2] by treatment of podophyllotoxin with phosphorus tribromide or thionyl bromide, the hydroxy-group being thereby replaced by a bromine atom. This "podophyllotoxin bromide" was reported to melt at 157.5—159° C, to give an immediate precipitate of silver bromide with alcoholic silver nitrate and to be rapidly hydrolyzed by water.



I Podohyllotoxin (PT)
trans-1:2-*trans*-2:3-*cis*-3:4
II Picropodophyllin (PP)
trans-1:2-*cis*-2:3-*trans*-3:4

III α-Peltatin
trans-2:3-*cis*-3:4

IV β-Peltatin
trans-2:3-*cis*-3:4

Fig. 1.

Further allusion to the subject is found in a paper by Hartwell and Detty [3], in which it is stated that podophyllotoxin and the peltatins (III and IV) are unreactive in the usual bromine test for unsaturation [4] and that the methyl ethers of the peltatins substitute bromine with evolution of hydrogen bromide.

In a recent short communication [5] we have reported that podophyllotoxin dissolved in chloroform and shaken with an aqueous bromine solution at room temperature virtually consumes approximately 2 equivalents of bromine per molecule. A solvate of the composition $C_{22}H_{22}O_8$, $\frac{1}{2}C_6H_6$, H_2O was used for these quantitative experiments.

The experimental equivalent weights varied with the excess of bromine and with the reaction time in a poorly reproducible manner, and the method proved inadequate for quantitative determination of podophyllotoxin. Under certain conditions, specified in the experimental section, equivalent weights in the neighbourhood of 235 were obtained. The calculated value is 235.5 on the assumption that 2 equivalents of bromine are consumed, one of which enters the podophyllotoxin molecule, the other one forming hydrogen bromide. When the reaction time was extended and/or when a larger excess of bromine was employed, considerably lower equivalent weights were obtained, probably indicating the substitution of a second hydrogen atom in the podophyllotoxin molecule.

Under the conditions leading to monosubstitution of podophyllotoxin picropodophyllin also gave equivalent weights fairly close to the value for monosubstitution. In both cases we have succeeded in isolating the monobromoderivative.

Monobromo-podophyllotoxin was described in a preliminary communication [5] and the data are given in the experimental section in the present paper. Although one of its melting points (155° C) is close to the melting point of Hartwell and Schrecker's podophyllotoxin bromide (157.5—159° C)[2], there is clear evidence that our product is a different compound. Contrary to podophyllotoxin bromide our bromo-derivative gave no precipitate of silver halide with silver nitrate even when heated, and the bromine was not split off in boiling alcoholic sodium hydroxide. It is obvious, therefore, that

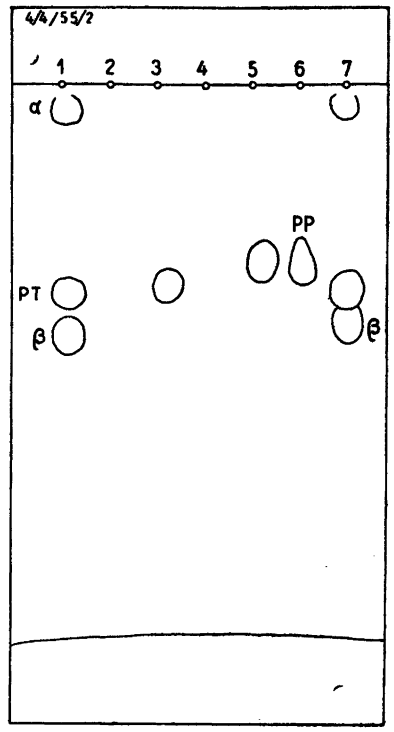


Fig. 2. Paper chromatogram, formamide-paper/benzene, 25.0°; general technique vide *Ref.*[16] *spray-reagent antimony pentachloride.*

1. Standard podophyllin, showing reference spots of α-peltatin, (α), podophyllotoxin (PT) and β-peltatin (β).
3. Authentic podophyllotoxin.
5. Product obtained on hydrogenolysis of bromo-podophyllotoxin.
6. Authentic picropodophyllin.
7. As No. 1.

the bromine atom is linked to an aromatic ring. That the hydroxy-group of podophyllotoxin (I) is intact in our derivative is compatible with the elementary analysis and unequivocally proved by the existence of an acetyl derivative and by the observation that the bromo-derivative may be hydrogenated with Raney-Ni catalyst to picropodophyllin, the bromine-atom being thereby removed and the resulting podophyllotoxin epimerized in the alkaline solution. The identity of the hydrogenation product with authentic picropodophyllin was proved by melting point and paper chromatography, Fig 2.

The exact position of the bromine atom in monobromo-podophyllotoxin remains to be discussed. According to the above observations the bromine atom may have entered either ring A or ring C (I). It was thought that an oxidative degradation of the molecule would distinguish between these possibilities. Permanganate oxidation of bromo-podophyllotoxin may be expected to proceed as indicated for the parent compound in Fig. 3, bromo-derivatives of (V), (VI) or (VII) being formed, depending upon the position of the bromine atom and the experimental conditions. By exhaustive permanganate oxidation of podophyllotoxin at 100° in weakly alkaline solution Späth *et al.*[6] obtained trimethoxybenzoic acid (VI) in good yield, whereas the rings A, B and D were completely broken down to oxalic acid. Shortly afterwards similar results were reported by Borsche and Niemann[7]. By an

Fig. 3. Oxidative degradation of podophyllotoxin [6-10]

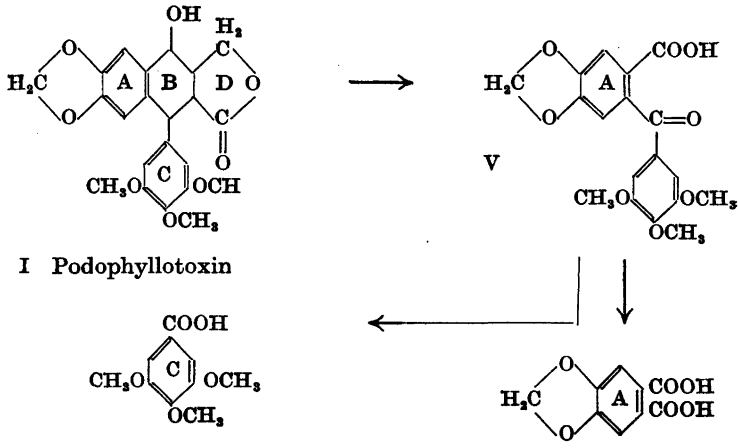
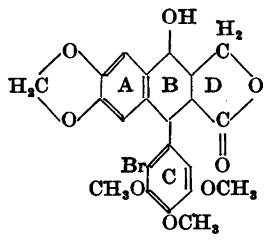


Fig. 4. Bromo-podophyllotoxin.



oxidation carried out under milder conditions (50° C) Späth *et al.* [8] later obtained the intermediate keto-acid (V), the structure of which they proved by synthesis. It is of no consequence in this connection that their starting material was picropodophyllin, since this compound differs from podophyllotoxin only in the configuration of ring B. Further oxidative degradation of (V) would be expected to give a mixture of (VI) and (VII). Hydrastic acid (VII) was isolated in a very small quantity from permanganate oxidation of podophyllotoxin at 60—70° C by Späth *et al.* [9].

Degradation products closely analogous to (V), (VI) and (VII) have been isolated from a permanganate oxidation of the peltatins and their ethers by Schrecker and Hartwell [10], *cf.* [3]. The general scheme of reactions in Fig. 3 is therefore well established.

On oxidation of bromopodophyllotoxin with excess of permanganate at 100° C we have obtained a crystalline substance, which proved to be monobromo-trimethoxybenzoic acid (monobromo-VI). This finding settles the question as to the position of the bromine atom; it has substituted one of

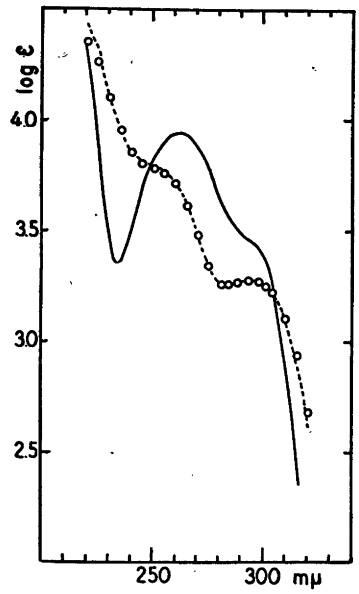


Fig. 5. Ultraviolet absorption curves (96 % ethanol). ——— *3,4,5-trimethoxybenzoic acid.* ----- *2-bromo-3,4,5-trimethoxybenzoic acid.* O *Degradation product from bromopodophyllotoxin.*

the remaining hydrogen atoms at ring C (Fig. 4) and has not entered ring A, as we tentatively suggested in our preliminary communication [5].

The identity of the degradation product was established in the following way. Its melting point was 151—152° (lit. 151—152°, Ref. [11, 12]) and it did not depress the melting point of an authentic sample, prepared synthetically. Its ultraviolet absorption curve was identical with the curve of the synthetic bromo-derivative but distinctly different from that of the parent compound, trimethoxybenzoic acid itself, Fig. 5.

From an oxidation carried out under milder conditions (55°) two degradation products were obtained. One of these, which did not contain bromine, came out in a quantity too small for complete purification. It has not been definitely identified but is believed to be hydrastic acid. Its melting point was 175° C; hydrastic acid is reported to melt at 173—187° or higher, depending upon the rate of heating, more or less anhydride being formed during the melting process [13].

The second product isolated from the "mild degradation procedure", provisionally named 19/10/54/I, analyzed as bromopodophyllotoxin, but had a much higher melting point, 225—229° C. It would be a reasonable guess therefore, that it consisted of bromo-picropodophyllin, the epimer of the non-oxidized starting material, the formation of which could be anticipated, since the oxidation mixture becomes weakly alkaline during the process. For the purpose of comparison authentic bromo-picropodophyllin was therefore prepared both by bromination of picropodophyllin and by base-catalyzed, inversion of bromo-podophyllotoxin. The latter two compounds proved to be identical with respect to optical rotation and the R_F-value on paper-chromatograms, Fig. 6. Their ultra-violet absorption curves (Fig. 7, —·—·

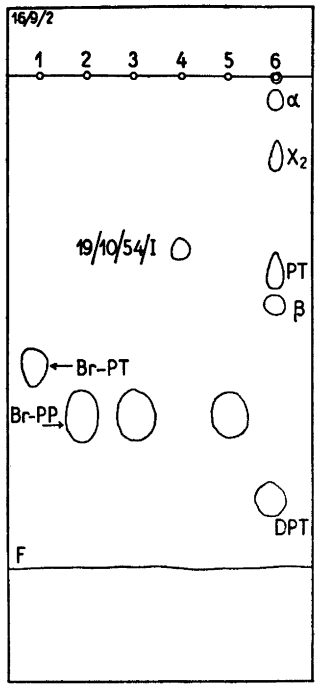


Fig. 6. Paper chromatogram, formamide-paper/benzene, 25.0° C. Spray-reagent antimony pentachloride (general technique vide *Ref.*[16])

1. *Bromopodophyllotoxin (Br-PT) by bromination of podophyllotoxin.*
2. *Bromo-picropodophyllin (Br-PP) by inversion of bromopodophyllotoxin.*
3. *Bromo-picropodophyllin by bromination of picropodophyllin.*
4. *Substance 19/10/54/I isolated from degradation of bromopodophyllotoxin.*
5. *As 2.*
6. *Standard podophyllin, showing reference spots of α-peltatin (α) podophyllotoxin (PT), β-peltatin (β) and desoxy-podophyllotoxin (DPT).*

and ———) showed qualitative agreement. The product obtained by bromination is probably not quite as pure as that prepared by inversion. Unfortunately they both failed to crystallize, a comparison by melting points thus being prevented.

From the ultraviolet absorption curve of the substance 19/10/54/I (Fig. 7, O) it can only be inferred that it is closely related to bromo-picropodophyllin or bromo-podophyllotoxin, the curves of which are almost identical. The available quantity was insufficient for measurement of the optical rotation. Paper-chromatography gave the surprising result that the substance had a considerably lower R_F-value than the two authentic specimens of bromo-picropodophyllin. Fig. 6, No. 4, is representative of a large number of chromatograms, which all showed the same relative location, far above the spots of bromo-podophyllotoxin and bromo-picropodophyllin. This also implies that the substance cannot be merely a new crystal modification of bromo-podophyllotoxin. The possibility that the mild permanganate oxidation had caused a dehydrogenation at ring B is ruled out, since the resulting compound, bromo-dehydropodophyllotoxin, would in all probability have an ultraviolet absorption curve similar to that of dehydropodophyllotoxin (Fig. 8, *cf.* Ref. [15]) and this is seen to be entirely different from the curve of the substance in question (Fig. 7, O). The substance 19/10/54/I may possibly have the structure indicated in Fig. 9; the parent compound podophyllotoxone (and picropodophyllone, respectively) has recently been obtained by Gensler

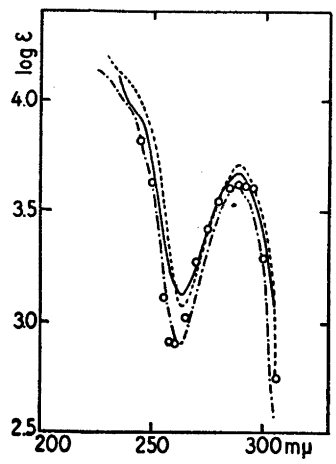


Fig. 7. Ultraviolet absorption curves (96 % ethanol). ----- *bromopodophyllotoxin.* ——— *bromo-picropodophyllin, by bromination of picropodophyllin.* —·—·—· *bromo-picropodophyllin, by inversion of bromopodophyllotoxin.* O *Substance 19/10/54/I isolated from degradation of bromopodophyllotoxin.*

and Johnson [18] by oxidation of podophyllotoxin and picropodophyllin, respectively, with freshly prepared manganese dioxide, *i.e.* under conditions very similar to ours. The analytical figures for bromo-podophyllotoxone (Calc. for $C_{22}H_{19}O_8Br$: C 53.8; H 3.9; Br 16.3; $3OCH_3$ 18.9) fit fairly well with the experimental data. The ketonic structure would furthermore account for the anomalous location on paper chromatograms, and the ultraviolet absorption curve of substance 19/10/54/I has certain features in common with those

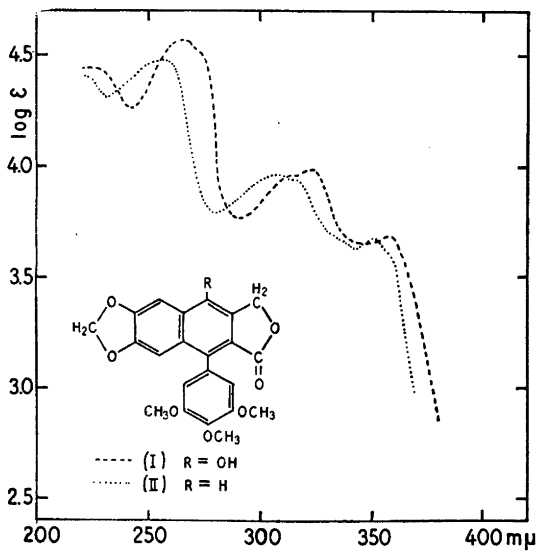


Fig. 8. Ultraviolet absorption curves (96 % ethanol). ---- *dehydropodophyllotoxin* [15] ····· *dehydroanhydro-picropodophyllin.*

reported for podophyllotoxone and picropodophyllone[18]. Further experiments in progress are hoped to produce conclusive evidence.

Finally it should be mentioned that we have not during our work isolated any substance corresponding to Dunstan and Henry's high-melting bromo-derivative, to which we referred at the outset.

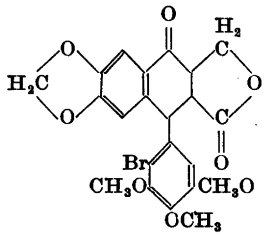


Fig. 9. Bromo-podophyllotoxone, bromo-picropodophyllone.

EXPERIMENTAL

All melting points are corrected and have been determined with the hot-stage microscope essentially according to Kofler.

Podophyllotoxin and picropodophyllin. The preparation and characterization were as reported in a previous communication[16] *cf.* [17]. Podophyllotoxin was a solvated product, analyzing as $C_{22}H_{22}O_8$, H_2O, $\frac{1}{2}C_6H_6$ (mol.wt. 471), whereas picropodophyllin was unsolvated $C_{22}H_{22}O_8$ (mol.wt. 414).

Bromination procedure. About 0.4 g of the substance was dissolved in 25 ml of chloroform in a 100 ml conical flask, provided with a ground glass stopper. 25.00 ml of 0.1 N potassium bromate (2.5 milliequivalents bromine), 1.0 g of potassium bromide and 10 ml of 4 N sulphuric acid were added and the closed flask was allowed to stand in the dark for 15 minutes with frequent agitation. Then 5 ml of potassium iodide solution (10 %) was added and the contents of the flask vigorously shaken. Upon standing for 5 minutes in the dark the reaction mixture was titrated with 0.1 N sodium thiosulphate, 5 ml 1 % starch solution being added towards the end of the titration.

With this standard procedure podophyllotoxin gave equivalent weights ranging from 220 to 238, calculated 235.5 for monosubstitution. With a larger excess of bromine and/or extended reaction time somewhat lower equivalent weights were obtained. Picropodophyllin similarly gave equivalent weights in the region 200 to 230, calculated 207 for monosubstitution.

The isolation of the bromination-products is described below.

Bromopodophyllotoxin. Five batches of bromination mixture prepared and titrated as above from a total of 2.0 g podophyllotoxin were used for the isolation of bromopodophyllotoxin. Every single batch showed a bromine consumption corresponding to monosubstitution. The separated aqueous layers were washed with chloroform, which was then added to the original chloroform layers. The combined chloroform volumes were washed with water, dried with anhydrous sodium sulphate and evaporated to dryness *in vacuo*. The residue was recrystallized from benzene and dried at 50° C. Yield 1.6 g of colourless needles, melting partly at 155° and partly at 183° C. Subsequent drying to constant weight at 130° and 0.01 mm Hg gave a loss of weight amounting to 10.0 % indicating that the substance was a solvate. The melting point 155° is most probably that of the solvate and is not due to a specific crystal modification of the unsolvated substance as tentatively suggested in the prodromal communication.[5] The melting point of the vacuum-dried sample was 182—183° C, and it analyzed as monobromopodophyllotoxin (Found: C 53.6, 53.8; H 4.4, 4.5; Br 16.3, 16.7; 3 OCH_3 18.7. Calc. for $C_{22}H_{21}O_8Br$. (493.3): C 53.5; H 4.3; Br 16.2; 3 OCH_3 18.9). $[\alpha]_D^{20} = -92°$ (*c* 1.0, chloroform); $[\alpha]_D^{20} = -73°$ (*c* 0.90, ethanol).

The substance gave no precipitate with alcoholic silver nitrate even on heating, and alcoholic sodium hydroxide boiled with bromopodophyllotoxin gave a negative test for bromide ion.

Acetyl-bromopodophyllotoxin. A mixture of 0.20 g of bromopodophyllotoxin and 5 ml of acetic anhydride was refluxed for two hours. Excess of the reagent was decomposed at room temperature with 10 ml of water. The isolated solid was recrystallized from benzene and dried to constant weight at 120° C and 0.01 mm Hg (18 h). Colourless needles melting at 217–221° C (Found: C 53.7; H 4.6; Br(16.0); 3 OCH_3 17.9. Calc. for $C_{24}H_{23}O_9Br$ (535.3): C 53.8; H 4.3; Br 14.9; 3 OCH_3 17.4). $[\alpha]_D^{20} = -93°$ (*cf.* 0.88; chloroform).

Bromopicropodophyllin (by bromination of picropodophyllin). By bromination and isolation as described for bromopodophyllotoxin above, an almost colourless amorphous solid was obtained. Attempts to bring it into the crystalline state proved unsuccesful. A sample, which had been dried to constant weight at 100° C and 0.01 mm Hg, analyzed as unsolvated bromopicropodophyllin (Found: C 53.6; H 4.4; Br 15.9; Calc. for $C_{22}H_{21}O_8Br$ (493.3): C 53.5; H 4.3; Br 16.2; 3 OCH_3 18.9). Paperchromatographic examination (Fig. 6) and the ultraviolet absorption spectrum (Fig. 7) as well as the optical rotation, $[\alpha]_D^{20} = -17.5°$ (c = 1.09; chloroform), strongly suggest that the substance is identical with bromopicropodophyllin, obtained by inversion of bromopodophyllotoxin (*vide infra*). The amorphous substance failed to give a crystalline acetyl derivative.

Bromopicropodophyllin (by inversion of bromopodophyllotoxin).

A solution of 1.0 g of bromopodophyllotoxin and 0.2 g of anhydrous sodium acetate in 20 ml of 99 % ethanol was refluxed for 18 hours. The reaction mixture was allowed to cool to room temperature, 40 ml of water was added, and the solution was extracted with 2 × 50 ml of chloroform. The combined chloroform layers were dried with anhydrous sodium sulphate and evaporated to dryness in vacuo. The almost colourless residue did not crystallize and showed an undefined melting point. $[\alpha]_D^{20} = 16.5°$ (c 1.03; chloroform).

The ultraviolet absorption spectrum (Fig. 7) shows the essential features common to bromopodophyllotoxin and the bromination product of picropodophyllin. A paperchromatographic examination (Fig. 6) clearly indicates identity with the latter and non-identity with the former compound.

Hydrogenation of bromopodophyllotoxin. 100 milligrams of bromopodophyllotoxin were dissolved in 5 ml of 99 % ethanol, 0.1 g of Raney-nickel catalyst and 5 ml of 2N sodium hydroxide were added and the mixture was refluxed on a steam bath for 20 minutes. The solution was diluted with 10 ml of water, acidified with sulphuric acid and shaken out with two times 20 ml chloroform. The combined chloroform phases were dried with anhydrous sodium sulphate and evaporated to dryness *in vacuo*. The residue was recrystallized from 99 % ethanol and yielded a few milligrams of colourless needles, m.p. 223–224° C. The sample did not depress the melting point of an authentic specimen of picropodophyllin and the identity was further evidenced by paper-chromatographic comparison (Fig. 2).

Permanganate oxidation of bromopodophyllotoxin at 100° C. 1.0 g bromopodophyllotoxin was dissolved in 20 ml of 1*N* sodium hydroxide and 10 ml of aceton. The solution was placed in a 400 ml beaker, fitted with a mechanical stirring device, and heated on the steam bath. A 4 % potassium permanganate solution was added by 5 ml batches with continuous stirring, no batch being added until the preceding one was decolourized. After addition of 150 ml of permanganate solution the aceton was distilled off and more permanganate was added until the colour became persistent (additional 100 ml were required).

The mixture was cooled to room temperature, filtered, concentrated to 25 ml and again filtered. The filtrate was acidified to Congo red. After 15 minutes a colourless crystalline solid deposited, yield 25 mg, m.p. 146–151°. Vacuum sublimation raised the melting point to 151–152° C. It analyzed as bromo-trimethoxybenzoic acid. (Found: C 41.5; H 3.9; Br 27.6; 3 OCH_3 32.2. Calc. for $C_{10}H_{11}O_5Br$ (291.1): C 41.3; H 3.8; Br 27.5; 3 OCH_3 32.0).

Its identity with 2-bromo-3,4,5-trimethoxybenzoic acid was proved by comparison of its ultraviolet absorption spectrum with that of a synthetic sample (Fig. 5).

Permanganate oxidation of bromopodophyllotoxin at 55° C. 500 milligrams of bromopodophyllotoxin were dissolved in 20 ml of aceton. 11 ml 4 % potassium permanganate solution was slowly run in with continuous stirring at 55° C. After heating for 30 minutes the colour had vanished, the aceton was distilled off, and the manganese dioxide was removed with sulphur dioxide gas. The solution was subsequently acidified to Congo red, extracted with chloroform, the combined chloroform extracts dried and evaporated to dryness *in vacuo*. The residue was dissolved in a mixture of 5 ml of 99 % ethanol and 5 ml of benzene and chromatographed on 10 g of "Aluminiumoxyd neutral, Woelm", the same solvent mixture being used as an eluent. One eluate amounting to 50 ml was collected. By evaporation of the solvents an almost colourless crystalline product (substance 19/10/54/I) was obtained, yield 30 mg, m.p. 225—229° C. It analyzed as bromopicropodophyllin (or bromopodophyllotoxin). (Found: C 53.9; H 4.2; Br (19.0); 3 OCH_3 19.3. Calc. for $C_{22}H_{21}O_8Br$ (493.3): C 53.5; H 4.3; Br 16.2; 3 OCH_3 18.9).

3,4,5-Trimethoxybenzoic acid. The authentic specimen was prepared by methylation of gallic acid according to "Organic Syntheses"; m.p. 167° C, lit. 167° C.

2-Bromo-3,4,5-trimethoxybenzoic acid. 5.0 g of trimethoxybenzoic acid was dissolved in 40 ml of chloroform. A solution of 3.8 g of bromine in 10 ml of chloroform and some iron filings were added. The mixture was refluxed for 4 hours. The solvent was distilled off *in vacuo* and the residue was recrystallized from 400 ml of water, active carbon being added for decolouration. Yield 4.0 g of colourless needles, m.p. 151—152°. lit.[12] 148° C.

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A Preparative, Refractometric and Spectrophotometric Investigation of Some Phenoxysilanes

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The synthesis of phenoxyalkoxysilanes by various methods was studied. The refractometric properties and ultraviolet absorption spectra of the compounds prepared were measured. From the results obtained it was possible to draw certain conclusions concerning the charge distribution in the phenoxysilanes.

As an extension of previous studies of various types of silanes and siloxanes [1] an investigation of some phenoxysilanes, including phenoxymethoxysilanes, phenoxyethoxysilanes and tetraphenoxysilane from a preparative, refractometric, and spectrophotometric point of view is now reported.

PREPARATION OF PHENOXYSILANES

Several methods were tried. The first one involved the reaction between a phenoxychlorosilane and an alcohol, the second a controlled disproportionation of a phenoxyalkoxysilane, and in the third a tetraalkoxysilane was allowed to react with phenol in the presence of sodium phenoxide as a catalyst.

Method 1. Chlorosilanes usually react easily with alcohols with the exchange of alkoxy groups for chlorine. Thus the principal reaction product between a phenoxychlorosilane and an alcohol might be expected to be the corresponding phenoxyalkoxysilane. However, if the synthesis is made in the absence of an acceptor for hydrogen chloride this is by no means the case. Table 1 summarizes some preparations according to method 1. In run No.1, mainly phenoxytrimethoxysilane and tetraphenoxysilane were formed in a reaction between diphenoxydichlorosilane and methyl alcohol. Thus a considerable disproportionation took place.

The effect of a base is demonstrated in run No. 2. Here the synthesis in No. 1 was repeated using pyridine as an acceptor for the hydrogen chloride. Disproportionation was diminished appreciably and the yield of the required diphenoxydimethoxysilane increased from 5 to 47 %. To obtain a reasonable yield of triphenoxymethoxysilane from triphenoxychlorosilane and methyl

Table 1. Preparation of phenoxymethoxysilanes and tetraphenoxysilane from phenoxychlorosilanes and methyl alcohol.

No.	Starting material (mole)	Products **				Conditions
		$C_6H_5OSi(OCH_3)_3$	$(C_6H_5O)_2Si(OCH_3)_2$	$(C_6H_5O)_3SiOCH_3$	$(C_6H_5O)_4Si$	
1 *	$(C_6H_5O)_2SiCl_2$(0.14)+CH_3OH (0.31)	51	5	2	33	Without solvent. Refluxing for 20 min. after addition of the alcohol.
2 *	$(C_6H_5O)_2SiCl_2$(0.14)+CH_3OH (0.31)+C_5H_5N(0.31)	12	47	4	8	Solvent carbon tetrachloride. Temp. 50—60°.
3	$(C_6H_5O)_3SiCl$(0.10)+CH_3OH (0.12)+C_5H_5N(0.12)	5	30	12	40	As in No. 2.
4	$(C_6H_5O)_3SiCl$(0.10)+CH_3OH (0.12)+C_5H_5N(0.12)	2	16	54	22	Solvent carbon tetrachloride. Temp. 0—4°.

* Some tetramethoxysilane was also formed in these syntheses.
** Mol.-% calculated on the chlorosilane used as starting material.

Table 2. Preparation of phenoxyalkoxysilanes and tetraphenoxysilane from tetraalkoxysilanes or phenoxytriethoxysilane and phenol.

No.	Starting material(mole)	Products *					Conditions
		$(RO)_4Si$	$C_6H_5OSi(OR)_3$	$(C_6H_5O)_2Si(OR)_2$	$(C_6H_5O)_3SiOR$	$(C_6H_5O)_4Si$	
1	$(CH_3O)_4Si$(0.5)+C_6H_5OH(0.5)	61	16	4	1	18	Sodium phenoxide as catalyst
2	$(C_2H_5O)_4Si$(0.5)+C_6H_5OH(0.5)	26	56	4	0	7	—»—
3	$C_6H_5OSi(OC_2H_5)_3$(0.2)+C_6H_5OH (0.2)	0	34	33	1	25	—»—
4	$(C_2H_5O)_4Si$(0.3)+C_6H_5OH(0.1)	10	71	13	0	0	—»—
5	$(C_2H_5O)_4Si$(0.3)+C_6H_5OH(0.1)	80	13	0	0	0	Without catalyst

* Mol.-% calculated on the silane used as starting material. In Nos. 4 and 5 the yield was calculated on 0.1 mole of the silane. R denotes methyl or ethyl depending on the starting material.

alcohol, however, it was not sufficient to absorb the hydrogen chloride but also necessary to carry out the reaction at a low temperature (*cf.* runs Nos. 3 and 4). A possible explanation of this fact will be given later in this paper.

Method 2. To examine the cause of the disproportionation of the phenoxyalkoxysilanes in the syntheses above some experiments were carried out. It was found that phenoxyalkoxysilanes could be heated, without appreciable change, in the presence of dry hydrogen chloride or small amounts of an alcohol or water. The simultaneous presence of hydrogen chloride and a hydroxylic compound, however, caused a disproportionation *. Furthermore it was found that the disproportionation could be controlled by using suitable conditions (see the experimental part). Thus it was possible from one phenoxyalkoxysilane to prepare the two "neighbours".

$$(C_6H_5O)_nSi(OR)_{4-n} \rightleftharpoons (C_6H_5O)_{n-1}Si(OR)_{5-n} + (C_6H_5O)_{n+1}Si(OR)_{3-n} \quad (1)$$

Method 3. The reaction between tetramethoxysilane and tetraethoxysilane, respectively, and phenol in equimolecular quantities and in the presence of sodium phenoxide is demonstrated by runs Nos. 1 and 2 of Table 2. The fact that a considerable part of the tetraalkoxysilanes remains after completion of the reaction and the formation of substantial amounts of tetraphenoxysilane indicates a ready reaction of the phenoxyalkoxysilanes with the phenoxide ion under the prevailing conditions. The same conclusion may be drawn from run No. 3, where phenoxytriethoxysilane and phenol were allowed to react in the molar ratio 1:1. Furthermore from the figures in runs Nos. 1, 2, and 3 it is evident that the reactivity of a silane $(C_6H_5O)_nSi(OR)_{4-n}$ increases when n increases from 1 to 3 and is greater for $R = CH_3$ than for $R = C_2H_5$. The low yield of triphenoxyalkoxysilanes in all instances is noteworthy. Obviously these compounds, when formed, immediately react with phenoxide ion to tetraphenoxysilane.

To get a good yield of a particular phenoxyalkoxysilane from the alkoxysilane with one phenoxy group less, and phenol, it was necessary to use the alkoxysilane in excess (*cf.* run No. 4). A suitable inert diluent should also serve. In this way the formation of higher phenoxyalkoxysilanes was suppressed to a great extent. In the absence of a catalyst the exchange of phenoxy groups for alkoxy groups proceeded slowly and with a low yield of phenoxyalkoxysilanes (*cf.* run No. 5) **.

The boiling points and analyses of the compounds prepared in this investigation are given in Table 3.

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* Similar experiences were made by Peppard *et. al.*[3] with mixed tetraalkoxysilanes.

** A new procedure for the alkoxylation of halosilanes using alkyl orthoformates was recently described by Shorr [3]. An attempt was made to prepare diphenoxydiethoxysilane by this method. However, an impure product was obtained (see the experimental part).

EXPERIMENTAL

Phenoxymethoxysilanes from phenoxychlorosilanes and methyl alcohol (Method 1).

Phenoxychlorosilanes. Jörg and Stetter [4] prepared diphenoxydichlorosilane and triphenoxychlorosilane by adding silicon tetrachloride to molten phenol and then heating the mixture. They attempted, without success, to prepare phenoxytrichlorosilane by this method. Thompson and Kipping [5] added a solution of phenol in benzene to boiling silicon tetrachloride whereupon the mixture was heated to 200° for three to four hours. By this method all three of the phenoxychlorosilanes were obtained. The diphenoxy- and triphenoxychlorosilanes were obtained by Vol'nov [6] in a reaction between phenyl acetate and silicon tetrachloride. A modification of the Thompson and Kipping procedure was used in the work described in this paper.

Phenol (23.5 g, 0.25 mole) was dissolved in 60 ml of dry toluene and the solution added to 42.5 g (0.25 mole) of silicon tetrachloride with rapid stirring. The mixture was heated to 100° for one hour, the toluene and some unchanged silicon tetrachloride were removed and the residue fractionated in vacuum. There was obtained 25.0 g (44 %) of phenoxytrichlorosilane, 10.5 g (15 %) of diphenoxydichlorosilane and 6.3 g (7 %) of triphenoxychlorosilane. The yield of the various phenoxychlorosilanes is dependent on the ratio of phenol to silicon tetrachloride, the amount of solvent used, and the heating time. By using suitable conditions it is possible to obtain any of the phenoxychlorosilanes as the principal product.

The boiling points of the phenoxychlorosilanes were: phenoxytrichlorosilane 70° (9 mm), diphenoxydichlorosilane 156–157° (13 mm), and triphenoxychlorosilane 210–212° (9 mm). Their purity was ascertained by chlorine analyses.

Phenoxymethoxysilanes (Table 1). In run No. 1 the methyl alcohol was dropped into diphenoxydichlorosilane and the mixture heated to 60–70° for 20 minutes. Fractionation yielded tetramethoxysilane, phenoxymethoxysilanes and a residue of tetraphenoxysilane which was recrystallized from petroleum ether.

When pyridine was used as hydrogen chloride acceptor (runs Nos. 2 and 3) the phenoxychlorosilane was dissolved in carbon tetrachloride and the mixture of methyl alcohol and pyridine then added by stirring. The temperature rose to 50–60° during the addition. The precipitate of pyridine hydrochloride was filtered with the exclusion of moisture and washed with carbon tetrachloride; the solvent was distilled off at atmospheric pressure and the residue treated as above. In run No. 4 the heat of reaction was dissipated by cooling the reaction mixture with icewater during the addition of the methyl alcohol-pyridine mixture and the removal of the solvent was carried out at 30–35° (180 mm).

Disproportionation of phenoxyalkoxysilanes (Method 2).

As an example of the application of this method the preparation of phenoxytrimethoxysilane and triphenoxymethoxysilane from diphenoxydimethoxsysilane is described.

To 27.6 g (0.1 mole) of diphenoxydimethoxysilane was added one drop of methyl alcohol. A stream of dry hydrogen chloride was bubbled through the mixture with simultaneous refluxing for one hour. Fractionation furnished 4.3 g (0.02 mole) or 20 % of phenoxytrimethoxysilane, 13.8 g (0.05 mole) or 50 % of diphenoxydimethoxysilane, and 7.1 g (0.021 mole) or 21 % of triphenoxymethoxysilane. A small residue of tetraphenoxysilane remained in the distillation flask. If the conversion of the diphenoxydimethoxysilane is restricted to about 50 % almost no tetramethoxysilane and tetraphenoxysilane are obtained.

Phenoxysilanes from alkoxysilanes and phenol (Method 3)*.

The alkoxysilane and phenol were mixed and a small amount of sodium added which dissolved as sodium phenoxide. Slow distillation, about 8 hours per mole of alcohol,

* This method was previously used by Malatesta [7] for the preparation of tetraphenoxysilane from tetraethoxysilane and phenol.

Table 3. Boiling points and analyses.

Compound *	B. p. °C (mm)	% H		% Si	
		Found	Calcd.	Found	Calcd.
$C_6H_5OSi(OCH_3)_3$	102 (12)	6.61	6.59	13.2	13.11
$C_6H_5OSi(OC_2H_5)_3$	121 (12)	7.86	7.86	11.1	10.96
$(C_6H_5O)_2Si(OCH_3)_2$	160 (9)	5.89	5.84	10.2	10.16
$(C_6H_5O)_2Si(OC_2H_5)_2$	170 (9)	6.55	6.62	9.23	9.23
$(C_6H_5O)_3SiOCH_3$	140 (0.2)	5.41	5.36	8.23	8.30
$(C_6H_5O)_4Si$ **	—	5.10	5.03	6.94	7.01

* All compounds except tetraphenoxysilane are new.
** M. p. 52—53°.

Table 4. Densities, refractive indices and molar refractions.

Compound	Mol. wt	d_4^{20}	n_C^{20}	n_D^{20}	n_F^{20}	n_g^{20}	MR_C	MR_D	MR_F	MR_g
$C_6H_5OSi(OCH_3)_3$	214.29	1.1056	1.45767	1.46101	1.46944	1.47631	52.85	53.19	54.02	54.70
$C_6H_5OSi(OC_2H_5)_3$	256.37	1.0283	1.44943	1.45252	1.46027	1.46655	66.93	67.33	68.32	69.12
$(C_6H_5O)_2Si(OCH_3)_2$	276.36	1.1420	1.51018	1.51453	1.52562	1.53474	72.40	72.92	74.24	75.31
$(C_6H_5O)_2Si(OC_2H_5)_2$	304.41	1.0937	1.49999	1.50409	1.51448	1.52300	81.86	82.43	83.86	85.02
$(C_6H_5O)_3SiOCH_3$	338.42	1.1626	1.54340	1.54833	1.56136	1.57112	91.81	92.50	94.31	95.66
$(C_6H_5O)_4Si$	400.49	—	—	—	—	—	112.92	113.92	116.35	118.32

afforded an amount of alcohol equivalent to the amount of phenol used. The tetraalkoxysilanes and phenoxyalkoxysilanes were fractionated and the residual tetraphenoxysilane recrystallized from petroleum ether (see also Table 2).

Diphenoxydiethoxysilane from diphenoxydichlorosilane and ethyl orthoformate.

A mixture of 14.3 g (0.05 mole) of diphenoxydichlorosilane and 22.2 g (0.15 mole) of ethyl orthoformate was heated to 70° for one hour. Ethyl chloride was evolved. The ethyl formate formed and the excess of ethyl orthoformate were slowly distilled from the reaction mixture at atmospheric pressure and the residue fractionated in vacuum. There was obtained 2.2 g (17 %) of phenoxytriethoxysilane, b. p. 102—104° (5 mm), 9.5 g (62 %) of diphenoxydiethoxysilane, b. p. 152—154° (5 mm) and 3.0 g (17 %) of presumably triphenoxyethoxysilane, b. p. 196—197° (5 mm), $n_D^{20} = 1.535$. All of the products contained small amounts of chlorosilanes. Thus it appears that the alkoxylation of diphenoxydichlorosilane using this method is incomplete, at least under the conditions used here.

REFRACTOMETRIC INVESTIGATION

The phenoxyalkoxysilanes were redistilled to constant density and refractive index (n_D^{20}, Abbe-refractometer). For the samples thus obtained the refractive indices for the sodium D-line ($\lambda = 5\ 892.6$ Å), hydrogen C- and F-lines ($\lambda = 6\ 562.8$ Å and 4 861.3 Å, respectively), and mercury g-line ($\lambda = 4\ 358.3$ Å) were measured using a Bellingham and Stanley "Critical Angle Refractometer". These values together with densities and molar refractions (Lorenz-Lorentz-formula) are listed in Table 4. This Table also gives the molar refractions of tetraphenoxysilane. Since tetraphenoxysilane is a solid the molar refractions were determined using an indirect method.

Solutions of tetraphenoxysilane in *n*-heptane were made up and the refractive indices and densities of the solutions measured. From these values the specific refractions of the solutions (r_s) were calculated. The density and refractive indices of pure *n*-heptane were also measured and the specific refractions (r_h) calculated. The specific refractions of pure tetraphenoxysilane (r_x) were then obtained using the formula [8]

$$r_x = \frac{100r_s - (100-p)r_h}{p} \qquad (2)$$

assuming the specific refractions of tetraphenoxysilane and *n*-heptane to be additive * (p is the weight-% of tetraphenoxysilane in the solution). Multiplication of the r_x-values by the molar weight of tetraphenoxysilane gave the molar refractions.

For example, a solution of 0.5443 g of tetraphenoxysilane in 6.7968 g of *n*-heptane had $n_C^{20} = 1.39434$, $n_D^{20} = 1.39647$, $n_F^{20} = 1.40167$, $n_g^{20} = 1.40557$, and $d_4^{20} = 0.7064$. From these data the following specific refractions were calculated by the L.L.-formula: $r_C = 0.33888$, $r_D = 0.34050$, $r_F = 0.34445$, and $r_g = 0.34740$. Pure *n*-heptane had $n_C^{20} = 1.38599$, $n_D^{20} = 1.38787$, $n_F^{20} = 1.39274$, $n_g^{20} = 1.39633$, $d_4^{20} = 0.68387$, $r_C = 0.34346$, $r_D = 0.34495$, $r_F = 0.34879$, and $r_g = 0.35161$. Using eqn. 2 the following specific refractions were obtained for tetraphenoxysilane: $r_C = 0.2816$, $r_D = 0.2849$, $r_F = 0.2903$, and $r_g = 0.2948$.

* Several measurements at different concentrations indicated the validity of this assumption.

Table 5. Molar dispersions, specific refractions, specific dispersions and Abbe numbers.

Compound	$MR_F - MR_C$	$MR_g - MR_C$	r_d	r_{F-C}	A
$C_6H_5OSi(OCH_3)_3$	1.168	1.843	0.2482	106.5	39.2
$C_6H_5OSi(OC_2H_5)_3$	1.392	2.192	0.2626	105.4	41.7
$(C_6H_5O)_2Si(OCH_3)_2$	1.838	2.913	0.2639	135.2	33.4
$(C_6H_5O)_2Si(OC_2H_5)_2$	1.997	3.163	0.2708	132.5	34.8
$(C_6H_5O)_3SiOCH_3$	2.504	3.851	0.2733	154.5	30.5
$(C_6H_5O)_4Si$	3.43	5.40	—	—	—

In Table 5 the molar dispersions of the F—C and g—C lines are collected together with the specific refractions [r_D(L.L.-formula)], specific dispersions ($r_{F-C} = \frac{n_F - n_C}{d^{20}_4}.10^4$) and Abbe numbers ($A = \frac{n_D - 1}{n_F - n_C}$). The bond refractions and bond dispersions of a phenoxy group bonded to silicon in various types of phenoxysilanes are listed in Table 6.

In a previous communication [9] an identification scheme for organosilicon compounds, utilizing some refractometric constants, was proposed. Although it might be possible to identify phenoxyalkoxysilanes by chemical or spectrophotometric means it is considered of interest to include them in the refractometric identification system. As seen from the figures in Table 5 it is easy to distinguish between the mono-, di- and triphenoxyalkoxysilanes using the specific dispersions or Abbe numbers. The specific refractions are of less use

Table 6. Bond refractions and bond dispersions of the phenoxy group bonded to silicon.

Compound	$r\lambda(Si-OC_6H_5)$				$d\lambda_1-\lambda_2(Si-OC_6H_5)$	
	C	D	F	g	F-C	g-C
$C_6H_5OSi(OCH_3)_3$	28.10	28.34	28.92	29.41	0.820	1.304
$C_6H_5OSi(OC_2H_5)_3$	28.11	28.34	28.93	29.42	0.824	1.310
$(C_6H_5O)_2Si(OCH_3)_2$	27.95	28.18	28.75	29.23	0.803	1.277
$(C_6H_5O)_2Si(OC_2H_5)_2$	27.99	28.22	28.80	29.28	0.809	1.287
$(C_6H_5O)_3SiOCH_3$	27.85	28.07	28.65	29.08	0.796	1.224
$(C_6H_5O)_4Si$	28.23	28.48	29.09	29.58	0.86	1.35

Table 7. Differences between observed and calculated molar refractions and molar dispersions.

Compound	Δ_D	Δ_{F-C}
$C_6H_5OSi(OCH_3)_3$	−0.14	−0.040
$C_6H_5OSi(OC_2H_5)_3$	−0.14	−0.036
$(C_6H_5O)_2Si(OCH_3)_2$	−0.60	−0.114
$(C_6H_5O)_2Si(OC_2H_5)_3$	−0.52	−0.102
$(C_6H_5O)_3SiOCH_3$	−1.23	−0.192

Table 8. Molar extinction coefficients per phenoxy group.

λ(approx.)mμ max	$C_6H_5OSi(OC_2H_5)_3$	$C_6H_5OSi(OCH_3)_3$	$(C_6H_5O)_2Si(OC_2H_5)_2$	$(C_6H_5O)_2Si(OCH_3)_2$	$(C_6H_5O)_3SiOCH_3$	$(C_6H_5O)_4Si$
273	890	860	835	790	765	710
265	1 080	1 025	1 010	950	900	860
260	810	755	745	700	680	660

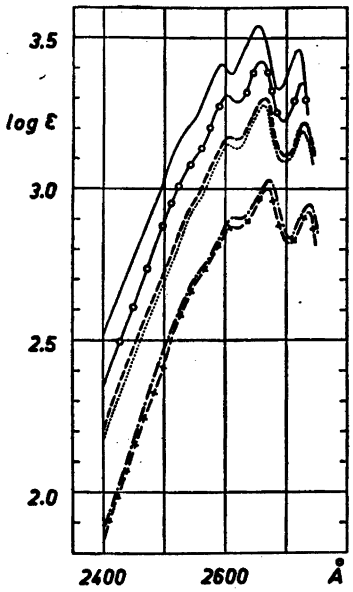


Figure 1. Absorption spectra of tetraphenoxysilane (————), triphenoxymethoxysilane (-O-O-O-), diphenoxydiethoxysilane (— — —), diphenoxydimethoxysilane (·····), phenoxytriethoxysilane (—·—·—), and phenoxytrimethoxysilane (— + — + —).

for this purpose. It is also possible to differentiate between the phenoxyalkoxysilanes and the eleven types of organosilicon compounds to which the refractometric identification method was previously applied (*cf.* Table 1, Ref[9].).

The bond refractions and bond dispersions of a phenoxy group bonded to silicon which are listed in Table 6 were, in the case of tetraphenoxysilane, obtained by dividing the molar refractions and molar dispersions of this compound by four and in the case of the phenoxyalkoxysilanes by subtracting the normal bond values of the alkoxy groups from the molar refractions and molar dispersions *. From the figures in Table 6 it is evident that in the phenoxyalkoxysilanes either the bond values of the phenoxy group or alkoxy groups or, which is most likely, of both of them are different from the normal ones. On that account the discrepancies between the observed molar refractions and molar dispersions and those calculated from the normal bond values are considerable (*cf.* Table 7).

When calculating molar refractions and molar dispersions of phenoxyalkoxysilanes the bond values of the phenoxy groups in Table 6 should be used together with the normal bond values of the alkoxy groups taken from Ref.[1] (*cf.* Tables 37, 38, 39, 48, 49, and 50 of this reference). Although Table 6 only

* The bond refraction of a group X bonded to silicon is written r(Si—X) and means the sum of the refraction of the group X and the bond Si-X. The normal value of the bond refraction r(Si-X) is defined as the bond refraction of the group in the silane SiX_n, where n equals 4 in the present case. The normal bond refraction is obtained by dividing the molar refraction of the silane SiX_4 by 4. Thus the normal bond refractions of the phenoxy group bonded to silicon are tabulated in the last row of Table 6. Concerning the bond dispersions d(Si-X) the same applies as to the bond refractions. The normal bond values of the alkoxy groups used in the calculation were taken from Ref.[1].

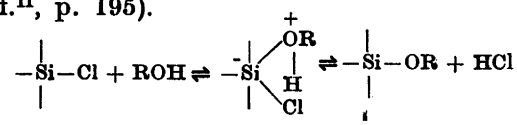
lists bond values of phenoxy groups in phenoxymethoxy- and phenoxyethoxysilanes it is believed that these values may also be used in calculating molar refractions and molar dispersions of phenoxyalkoxysilanes with other types of alkoxy groups.

SPECTROPHOTOMETRIC INVESTIGATION

The ultraviolet absorption of the phenoxysilanes was measured in *n*-heptane solution in the range 240—275 mμ using a Hilger UVISPEK spectrophotometer (see Fig. 1). The molar extinction coefficients per phenoxy group are given in Table 8. It is seen that in the series tetraphenoxysilane-triphenoxyalkoxysilane-diphenoxydialkoxysilane-phenoxytrialkoxysilane there is a bathochromic shift. Likewise a bathochromic shift exists between the corresponding phenoxymethoxysilanes and phenoxyethoxysilanes. The shifts are accompanied by hyperchromic effects as shown by the figures of the molar extinction coefficients per phenoxy group. The significance of the spectra will be further discussed below.

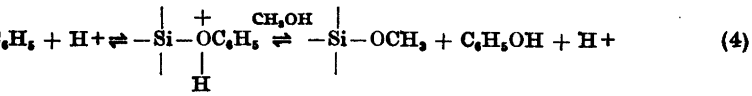
DISCUSSION

The reaction between a chlorosilane and an alcohol may be assumed to proceed in the same way as the reaction between an acid chloride and an alcohol * (*cf.* Ref.[11], p. 195).



(3)

A proof of the formation of intermediate addition compounds between chlorosilanes and alcohols has been given by Troost [12] who isolated adducts between silicon tetrachloride and alcohols at a low temperature. Similar addition compounds between silicon tetrafluoride and alcohols were reported by Gierut *et al.*[13]. In the absence of reactive groups other than chlorine at the silicon atom the only disturbance of the above reaction that may occur is hydrolysis of the chlorosilane or alkoxysilane by water formed in a side reaction between hydrogen chloride and the alcohol (*cf.* Ref.[1]). However, if other reactive groups are present (in the case of the phenoxychlorosilanes phenoxy groups) complications may arise as exemplified by synthesis No. 1 of Table 1. As pointed out previously the disproportionation is orginated by the simultaneous presence of hydrogen chloride and an alcohol. This fact indicates that the cause of the disproportionation is an acid-catalyzed re-esterification of the phenoxychlorosilane and/or phenoxyalkoxysilane.



(4)

If the phenol then reacts with another silane molecule than that from which it was formed, for instance by the exchange of a phenoxy group for a methoxy

* A similar mechanism was proposed by Swain *et al.*[10] for the hydrolysis of halogenosilanes which reaction was shown to proceed by a S_N2 attack by a water molecule on the halogenosilane.

group or a chlorine atom, this molecule gains one phenoxy group and the transference of a phenoxy group from one silane molecule to another is explained.

In the reactions between phenoxychlorosilanes and methyl alcohol using pyridine as a hydrogen chloride acceptor it was observed that the extent to which pyridine hindered the disproportionation in the case of the triphenoxychlorosilane was dependent on the temperature. A low temperature was necessary to obtain a reasonable yield of triphenoxymethoxysilane. No such temperature dependence was observed in the case of diphenoxydichlorosilane (*cf.* syntheses Nos. 2, 3, and 4, Table 1).

The function of pyridine or other tertiary amines in the reaction between halogenosilanes and alcohols has generally been considered to be merely that of a hydrogen chloride acceptor in spite of the fact that addition compounds between halogenosilanes and tertiary amines have been known to exist for a long time. [14, 15]. It is very likely, however, that the tertiary amine takes a more active part in the reaction and that the real "acylating" reagent is an addition compound between the amine and the chlorosilane *.

$$-\overset{|}{\underset{|}{Si}}-Cl + C_5H_5N \rightleftharpoons -\overset{|}{\underset{|}{Si}}-Cl,\ C_5H_5N \quad ** \qquad (5)$$

Such an assumption is supported by the observations made in this work and elsewhere ***. The stability of the addition compound should, among other things, be dependent on the structure of the chlorosilanes. From steric considerations the addition compound between pyridine and triphenoxychlorosilane might be expected to be less stable than that between pyridine and diphenoxydichlorosilane (*cf.* F-strain in amine salts [21]). If it be assumed that the extent to which pyridine hinders the disproportionation reaction is a function of the stability of its addition compound with the chlorosilane the influence of the temperature on the reaction between the phenoxychlorosilanes and methyl alcohol in the presence of pyridine is understandable.

The nucleophilic displacement of an alkoxy group by a phenoxy group was utilized in the third method used in this work for the preparation of phenoxyalkoxysilanes.

$$-\overset{|}{\underset{|}{Si}}-OR + C_6H_5O^- \rightleftharpoons -\overset{|}{\underset{|}{Si}}\begin{matrix}\diagup OC_6H_5 \\ \diagdown OR\end{matrix} \rightleftharpoons -\overset{|}{\underset{|}{Si}}-OC_6H_5 + RO^- \qquad (6)$$

$$RO^- + C_6H_5OH \rightleftharpoons ROH + C_6H_5O^- \qquad (7)$$

* It has been proposed that the promoting action of pyridine in benzoylation reactions is due to the formation of quaternary addition compounds between pyridine and benzoyl chloride (Ref. [11], p. 198). Furthermore acetylpyridinum chloride is a well known analytical reagent for the determination of active hydrogen in the hydroxyl groups of alcohols and phenols [16].

** The nature of the addition compounds is not definitely established. They have been formulated as co-ordination compounds in which use is made of vacant silicon orbitals, but also a quaternary salts [17-20].

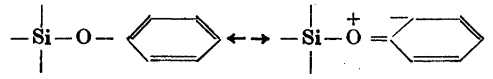
*** In the preparation of dimethyldialkoxysilanes and diphenyldialkoxysilanes from the corresponding chlorosilanes and alcohols, the reaction was incomplete in the absence of a tertiary amine. In the presence of pyridine, however, all of the chlorine atoms were replaced by alkoxy groups. The effect of pyridine can hardly be attributed to its salt formation with hydrogen chloride only, as other means of removing the hydrogen chloride *e. g.* boiling or blowing an inert gas through the reaction mixture were without effect [1].

From the figures of Table 2 it is apparent that the reactivity of phenoxyalkoxysilanes for phenoxide ion increases in the series

$$C_6H_5OSi(OR)_3 < (C_6H_5O)_2Si(OR)_2 < (C_6H_5O)_3SiOR$$

and is greater for R = CH_3 than for R = C_2H_5. This means that the positive charge on the silicon atom also increases in the mentioned series and is greater in the phenoxymethoxysilanes than in the corresponding phenoxyethoxysilanes. Such a charge distribution might be anticipated in view of the greater electron attraction of the phenoxy group in comparison with the alkoxy groups and the difference in inductive effects of the methyl and ethyl groups.

It is of interest that the difference in charge on silicon is also manifested in the absorption spectra of the phenoxysilanes. The ultraviolet absorption of these compounds originates from the aromatic part of the molecule. Mulliken [22] and others have stated from wave-mechanical considerations that absorption of light takes place predominantly through transitions to charge-resonance structures. Accordingly the transition energy is dependent on the resonance in the molecule. In the present case resonance occurs between such forms as *



The difference in energy between the ground state and the excited states is diminished with increased contribution of polar structures as a result of the lowering of the potential energy of the excited states. At the same time there is an increase in the molar extinction coefficients and bathochromic shifts take place [24].

The resonance in the phenoxysilane is dependent on the charge on the silicon atom in such a way that the contribution of polar structures decreases with increased positive charge **. Consequently from the study of the absorption curves in Fig. 1 and the molar extinction coefficients per phenoxy group in Table 8 it is apparent that the silicon atom in tetraphenoxysilane has the greatest positive charge which then decreases as alkoxy groups are exchanged for phenoxy groups. The greater positive charge on the silicon atom of the phenoxymethoxysilanes as compared with the corresponding phenoxyethoxysilanes is also clearly shown.

The differences between the molar refractions, calculated for the phenoxyalkoxysilanes from the normal bond refractions of the phenoxy and alkoxy groups and the molar refractions found, are appreciable*** (*cf.* Table 7). In

* To explain the acidity of the silanols R_3SiOH the participation of resonance structures such as $R_3Si{=}O^{-}H^{+}$ have been proposed [23]. In view of this resonance structures such as $Si{=}O^{-}{-}C_6H_5{}^{+}$ and $Si{=}O^{-}{-}R^{+}$ might contribute to the state of the phenoxyalkoxysilanes. At present there is no evidence, however, of the existence of such forms. While it seems unlikely that the contribution of the first type should be significant, the existence of the second might explain the variation of the apparent bond refraction of the phenoxy group bonded to silicon (see Table 6).

** Disturbances may arise, however, if steric hindrance is present [25].

*** Similar deviations were previously shown to exist for the alkylalkoxysilanes [1] [$RSi(OR)_3$(—0.10 ml), $R_2Si(OR)_2$ (—0.20 ml) and R_3SiOR(—0.10 ml)].

all probability this result has to be ascribed to the variation in charge on silicon. The differences increase as alkoxy groups are successively replaced by phenoxy groups indicating that a tightening of the Si—O bond of the alkoxy group is the main cause of the deviations (*cf.* note p. 1348).

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The System $CaSiO_3$-CaF_2

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The phase diagram of the system $CaSiO_3$—CaF_2 has been determined. From the depression of the freezing point it could be shown that, in the molten form, calcium metasilicate is polymerized by the condensation of three SiO_3^{2-} units probably in the form of a ring structure. Further, the activities of the components have been determined. The curves of the activity coefficients show some very interesting features.

The system $CaSiO_3$—CaF_2 has previously been studied by Karandéeff [1] and to a lesser extent by Tursky [2]. The former used the method of thermal analysis and found the system to be a simple one with eutectic point at 48 mole-% and 1 130° C (Fig. 1, dotted lines). The main difficulty was the undercooling at the silicate-rich side of the diagram. It is to be noticed, however, that recent determinations have given much higher melting points for the pure components. Tursky studied only a few melts in the microscope furnace and did not get satisfactory results due to reactions in the melts.

The present work utilizes the method of electric conductivity. In silicate-rich systems, this method will give no satisfactory results but, in CaF_2-rich mixtures, it is possible to obtain good values for the liquidus curve since CaF_2 is a crystallizer. The technique has been described in an earlier investigation [3] on the system CaO—CaF_2, from which the melting point of the fluoride, quoted below, has been taken. The melting point of pure $CaSiO_3$ is taken from the system CaO—SiO_2 [4]. It should be mentioned that no significant loss of fluoride occurs if the components of the mixtures are ignited and cooled before mixing, whereas 3—7 % will be lost when melting is performed without first driving off moisture.

The results have been recorded in Fig. 1. Dotted lines are from Karandéeff. The eutectic point was found at 41 mole-% $CaSiO_3$ and 1 127° C.

From measurements of the heat content of pure CaF_2, Naylor [5] has determined the heat of fusion, L_f = 7 100 cal/mole, and the heat capacities of the liquid and solid states. If we put the value for L_f into the expression for the freezing point depression together with the melting points of CaF_2 and CaF_2-

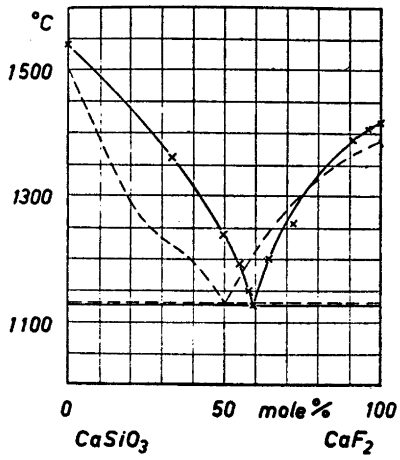

Fig. 1

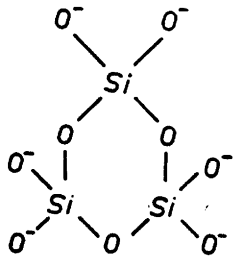

Fig. 2

rich mixtures, the mole fraction of solute can be computed. It is found that these values are only about one third of those given in Table 1, column 2. This indicates the association of three silicate units SiO_3^{2-}, *i. e.* the formula for the dissolved calcium metasilicate is $Ca_3Si_3O_9$. From X-ray investigations [6] it is known that crystallized calcium metasilicate, wollastonite, has a ring structure of just three SiO_3 units (Fig. 2). Probably this ring structure is also maintained in the pseudowollastonite existing above 1 150° C as well as in the molten state. This configuration has earlier been suggested by Bockris and Lowe [7] during the course of determinations of viscosities in molten Ca-silicates. They explain (see also Kozakevitch [8]) the decrease in viscosity of Ca-silicates, caused by addition of CaF_2, as a depolymerization action of the fluoride but from our results above such an explanation cannot be possible.

Taking into account the difference in molar heat of the solid and fused CaF_2, or ΔC_P, the activity of calcium fluoride in the melt can be computed using the formula

$$-\ln a_{CaF_2} = \frac{L_f \cdot \Delta T}{R \cdot T \cdot T_o} + \frac{\Delta C_P}{R}\left(\ln\frac{T_o}{T} + 1 - \frac{T}{T_o}\right)$$

Table 1.

Melting point °K	From weighing		Computed from freezing point depression		Apparent degree of polymerisation
	Mole fraction $CaSiO_3$	Mole $CaSiO_3$ per CaF_2	Mole fraction of solute	Mole solute per CaF_2	
1 691	0.000	0.0000	0.0000	0.0000	
1 680	0.040	0.0416	0.0137	0.0139	2.99
1 663	0.091	0.1000	0.0349	0.0362	2.76

Table 2.

Melting point °K	$x_{Ca_2Si_2O_6}$	x_{CaF_2}	$-\ln a_{CaF_2}$	a_{CaF_2}	$-\ln f_{CaF_2}$	f_{CaF_2}
1 691	0.000	1.000	0.000	1.000	0.000	1.000
1 680	0.0137	0.986	0.0138	0.986	0.000	1.000
1 663	0.0325	0.968	0.0356	0.965	0.0025	0.998
1 530	0.115	0.885	0.243	0.784	0.121	0.887
1 474	0.158	0.842	0.342	0.710	0.170	0.843
1 400	0.188	0.812	0.498	0.608	0.290	0.749

The results are summarized in Table 2. The composition will now be more adequately described by the mole fractions $x_{Ca_2Si_2O_6}$ and x_{CaF_2}, which are given in columns 2 and 3. The last two columns give the activity coefficients, $f = a/x$.

The activity coefficients can be expressed by the formula $-\ln f_{CaF_2} = 7.79 \cdot x^2_{Ca_2Si_2O_6}$, which using the Gibbs-Duhem formula yields $\ln f_{Ca_2Si_2O_6} = 15.58 \cdot x_{Ca_2Si_2O_6} - 7.79 \cdot x^2_{Ca_2Si_2O_6}$. Both activity coefficients are then put equal to unity in pure CaF^2, *i. e.* very dilute calcium silicate. For the eutectic mixture, where $x_{Ca_2Si_2O_6} = 0.188$, the values will be $-\ln f_{CaF_2} = 0.275$, $f_{CaF_2} = 0.759$ and $a_{CaF_2} = 0.617$ and also $\ln f_{Ca_2Si_2O_6} = 2.654$, $f_{Ca_2Si_2O_6} = 14.20$ and $a_{Ca_2Si_2O_6} = 2.67$.

We now consider the silicate-rich side of the diagram and need to know the heat of fusion for $Ca_3Si_3O_9$. This value can be computed from the system calcium metasilicate and diopside [9]. In this system $\ln x_{Ca_2Si_2O_6}$ turns out to be, within the experimental error, a linear function of $1/T$. This shows the liquid to be very nearly an ideal solution, and the heat of fusion for $Ca_3Si_3O_9$ comes out to be 40 800 cal/mole. As we do not know the heat capacities of solid and liquid silicate at the temperatures in question, we must disregard ΔC_p. There might be some solubility of Mg-silicate in the pseudowollastonite but Shairer and Bowen [9] consider it to be rather small. It is believed that the error in L_f arising in this way will have increased the value by at most two or three percent.

We now obtain the activity values summarized in Table 3. It is a strange coincidence that the activity coefficient for the silicate has very nearly the same value in the eutectic melt as in the pure silicate liquid.

Table 3.

Melting point °K	$x_{Ca_2Si_2O_6}$	x_{CaF_2}	$-\ln a_{Ca_2Si_2O_6}$	$a_{Ca_2Si_2O_6}$	$-\ln f_{Ca_2Si_2O_6}$	$f_{Ca_2Si_2O_6}$
1 813	1.000	0.000	0.000	1.000	0.000	1.000
1 633	0.403	0.597	0.624	0.536	0.287	1.333
1 513	0.250	0.750	1.122	0.326	0.265	1.304
1 466	0.214	0.786	1.338	0.263	0.206	1.229
1 423	0.195	0.805	1.550	0.212	0.083	1.087
1 400	0.188	0.812	1.669	0.189	0.003	1.003

Table 4.

Melting point °K	$x_{Ca_3Si_3O_9}$	x_{CaF_2}	$f_{Ca_3Si_3O_9}$	f_{CaF_2}
1 813	1.000	0.000	1.000	1.400
1 631	0.400	0.600	1.330	0.704
1 602	0.350	0.650	1.356	0.696
1 564	0.300	0.700	1.354	0.697
1 513	0.250	0.750	1.303	0.706
1 434	0.200	0.800	1.122	0.739
1 400	0.188	0.812	1.003	0.759
1 491	0.150	0.850	0.614	0.839
1 580	0.100	0.900	0.310	0.925
1 646	0.050	0.950	0.151	0.981
1 691	0.000	1.000	0.071	1.000

In computing the activity of the silicate in Table 3, we have used the pure substance as standard state, but in the formula for ln $f_{Ca_3Si_3O_9}$ above, we used the very dilute solution. The formula is renormalized giving $f = 1$ for pure silicate by substracting 2.65, which changes the value for the eutectic mixture 2.654 (see above) into 0.003 (Table 3). The values of f_{CaF_2} on the silicate side of the eutectic point are obtained by numerical integration of Gibbs-Duhem equation. The results are summarized in Table 4 and Fig. 3.

Here we can see that the activity coefficient of $Ca_3Si_3O_9$ rises to a maximum at about 33 mole-% and then decreases to a rather low value. It is interesting to notice that at the maximum there are two molecules of fluoride to one molecule of silicate.

In a pure silicate melt the divalent calcium ions will bind together the big *cyclo*-trisilicate ions by strong electrostatic forces and we suggest that this

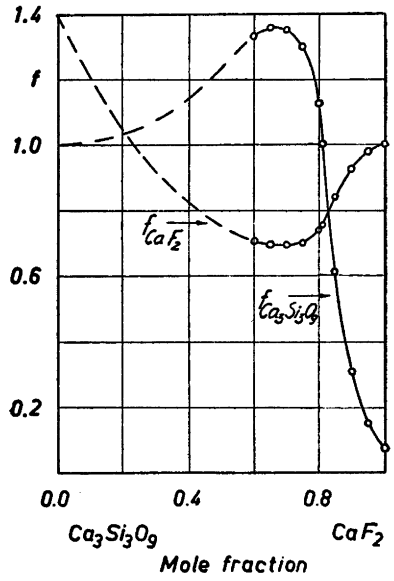

Fig. 3

is the cause of the high viscosity found by Herty *et al.*[10] and by Bockris and Lowe [7]. If we add calcium fluoride, the electrostatic binding will be broken because CaF^+ ion pairs are added to the large anions. We consider this to be the cause of the lower viscosity in the mixtures [10], but we find the substitution of O by F in the silicate ions, which has been proposed by other authors (*e. g.*[7,8]), to be rather improbable. Such a solvatization *e. g.* $Ca_3Si_3O_6 \cdot 3CaF_2$ will also explain the low activity coefficient of $Ca_3Si_3O_9$ diluted in CaF_2 and the low activity of CaF_2 in the same melts. In concentrated solutions with less CaF_2, the activity effects found might be explained by the stretching of the electrostatic bindings of the divalent Ca^{++} ions to the silicate ions by the added F^- ions and the corresponding compressing effect on the calcium fluoride.

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An Approximate Description of the π-Electron Distribution in Pyridine, Furan, and Pyrrole as Correlated with the Molecular Model, the Dipole Moment and the Chemical Properties of these Compounds

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By the use of a postulated relationship between bond-length and number of π-electrons in C,C, C,N, and C,O bonds a calculation of the electron distribution in molecular models for pyridine, furan, and pyrrole, consistent with a large amount of numerical data from microwave investigations, has been attempted. Care has been taken to choose models in harmony with experimental dipole moments and characteristic chemical properties.

In recent publications analyses of the microwave spectra of pyridine, furan, and pyrrole and several of their deuterated species were reported [1-3]. Because of difficulties in preparing the sufficient number of isotopic molecules no unequivocal determination of the structures was possible. A few (3,2 and 2, respectively) structural degrees of freedom were still left undetermined so that only a choice of models could be given. It is the purpose of this paper to show that between the possible structures *one* can be selected in each case, reproducing the measured dipole moment and predicting the well-known chemical properties of the substances.

THEORETICAL AND EMPIRICAL BACKGROUND.

The main procedure to be used in what follows is to draw conclusions as to π-electron "contents" of a chemical bond from its measured length. For C,C bonds the formula

$$\pi = \frac{2K}{K + \frac{x-d}{s-x}} = \frac{0.7150}{0.3575 + \frac{x-1.335}{1.540-x}} \qquad \text{(I)}$$

has been applied throughout. π is the number of π-electrons in the bond, x is the measured bond-length in Å, and 1.335 is the postulated distance for two atoms joined by a bond with 2 π-electrons. This number comes close to the experimental value for the length of the C,C, bond in ethylene but it need not coincide exactly with it. Also, the number 1.540 is the postulated bond-length in Å for *no* π-electrons. π-electron contents of C,O and C,N bonds are estimated by first adding 0.110, and 0.075 Å respectively to the experimental values and then using (I), a procedure which is in reasonable agreement with measured bond-lengths. The value of the constant $K = 0.3575$ was derived by letting 1.395 Å, the C,C distance in benzene, correspond to $\pi = 0.927\ e$. This is consistent with the generally accepted idea of benzene as a resonance hybrid between 78 % Kekulé-structures and 22 % Dewar-structures. The remaining $6 \times 0.073\ e$ are placed, not in the bonds, but on the carbon atoms [4]. Quite recently [5] Pritchard and Sumner have discussed the possible effects of changing K, d, and s in (I). They found that the numerical uncertainties in these parameters present no serious obstacle to the correlation of experimental and theoretical bond-lengths.

PYRIDINE

The resonance structures to be taken into account are (Fig. 1):

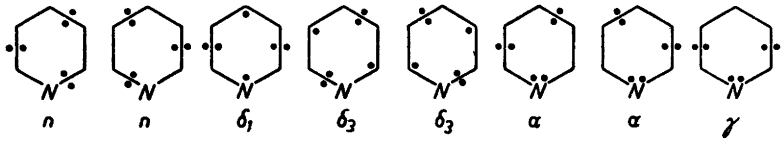

Fig. 1. Resonance components for pyridine (hydrogens omitted). Lines indicate σ-bonds, dots represent π-electrons. n, δ_1 etc. are "mole fractions" of resonance components.

In the case of pyridine it has already been demonstrated [1] that our model V (see Table 1) must be very nearly correct. By use of bond-lengths for this model and by means of (I) it is easily calculated that π(N,C) = 0.7027; π(C(2), C(3)) = 1.0256; π(C(3), C(4)) = 0.8924. Therefore, the total charge in the six bonds amounts to 5.241 e so that the total charge on the atoms must amount to 0.7586 e. By simply counting π-electrons in the various bonds it is now easy to derive the equations (III)—(V).

$$2n + \delta_1 + 2\delta_3 + 2\alpha + \gamma = 1.0000 \quad \text{(II)}$$
$$2n + 2\delta_3 = 0.7027 \quad \text{(III)}$$
$$2n + 2\delta_1 + 2\alpha + 2\gamma = 1.0256 \quad \text{(IV)}$$
$$2n + 2\delta_3 + 2\alpha = 0.8924 \quad \text{(V)}$$

It follows that n = 0.3103; $\alpha = 0.0948$; $\delta_3 = 0.0410$; $\gamma + \delta_1 = 0.1076$. Now, pyridine has a measured dipole moment of 2.26 $D\downarrow$. Approximately, this moment may be thought of as consisting of a σ-moment and a π-moment (from the π-electrons). The σ-moment has been estimated [6] to 0.85 $D\downarrow$ so that the π-moment is about 1.4 $D\downarrow$. We can utilize this by setting (VI):

$$1.41 = [0.704 \times 2\alpha + 2.813\,\gamma] \times 4.802 \quad \text{(VI)}$$

where 0.704 and 2.813 Å are the appropriate distances taken from model V. This gives $\gamma = 0.0578$. Since $\gamma + \delta_1 = 0.1076$ we get $\delta_1 = 0.0498$.

π-electron charges on the six ring-atoms may now be calculated. The charge on N is $\delta_1 + 4\alpha + 2\gamma$ which becomes 0.5446 *e*. The charges on C (2), C (3), and C (4) are 0.0410, 0.0410, and 0.0498 *e*, respectively. Accordingly, atomic and bond charges are distributed in pyridine as shown in Fig. 2.

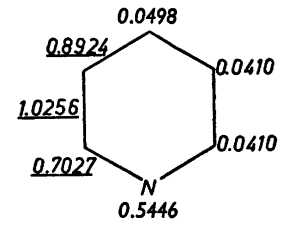

Fig. 2. Atomic and bond charges in pyridine (model V). Numbers are fractions of electronic charge. Bond charges have been underlined.

A discussion of how this model may be used in connection with a possible prediction of the fundamental chemical properties of pyridine is postponed to a following section.

FURAN

The resonance structures used are (Fig. 3):

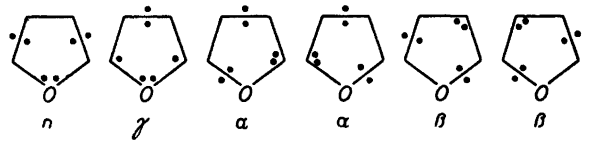

Fig. 3. Resonance components of furan (hydrogens omitted). Symbolism as for pyridine. $n + \gamma + 2\alpha + 2\beta = 1$ *(VII).*

The calculating procedure used above for pyridine is only applicable to some of the furan models given in Ref.[2]. For some of these models, negative values of, α,β *etc.* come out. Such models were discarded and a model looked for which permitted that the calculation could be carried through and also reproduced the dipole moment best possible. The finally selected model is characterized in Table 1 together with the corresponding models for pyridine and pyrrole here considered.

Table 1. Interatomic distances in the models of pyridine, furan and pyrrole applied at the calculations in this paper. Numbers in Å-units.

Distance:	Pyridine	Furan	Pyrrole
C,O		1.363	
C,N	1.342		1.387
C=C		1.362	1.367
C−C		1.442	1.428
C(2),C(3)	1.391		
C(3),C(4)	1.398		

Only models coming quite close to the models in table 1 fulfill the conditions mentioned.

Proceeding for furan as for pyridine it was found that $\pi(C,O) = 0.2958$, $\pi(C{=}C) = 1.4041$ and $\pi(C{-}C) = 0.4933$. This gives 3.8931 e in the bonds and 2.1069 e on the atoms. Furthermore

$$\begin{aligned} n + 2\alpha + 2\beta + \gamma &= 1.0000 && \text{(VII)}\\ 2\alpha + 2\beta &= 0.2958 && \text{(VIII)}\\ 2n + 2\beta &= 1.4041 && \text{(IX)}\\ 4\alpha + 2\gamma &= 0.4933 && \text{(X)} \end{aligned}$$

which gives $n = 0.6507$; $\alpha = 0.0966$; $\beta = 0.0513$; $\gamma = 0.0535$.

The experimental dipole moment of furan is 0.66 $D\uparrow$. The σ-moment has been estimated[6] to 1.30 $D\downarrow$. The expected π-moment is, therefore, 1.96 $D\uparrow$. Based on the model above one calculates a π-moment equal to $(0.1932 \times 0.826 + 0.1026 \times 2.127) \times 4.802 = 1.81$ $D\uparrow$ which is rather close to 1.96 $D\uparrow$ may be closer than could ordinarily be expected.

Accordingly, atomic and bond charges are distributed in furan as shown in Fig. 4.

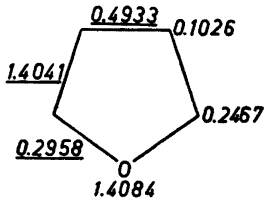

Fig. 4. Atomic and bond charges in furan (model in Table 1). Symbolism as in Fig. 2

A discussion of the chemical properties is postponed to a following section.

PYRROLE

The resonance structures considered are the same as for furan (Fig. 3). The model which could finally be selected between others that suffered from various defects has been described in Table 1. This model has $\pi(N,C) = 0.3724$; $\pi(C{=}C) = 1.3180$; $\pi(C{-}C) = 0.6020$, which means that there is 3.9828 e in the bonds and 2.0172 e on the atoms. The equations to solve are (XI—XIV):

$$\begin{aligned} n + 2\alpha + 2\beta + \gamma &= 1.0000 && \text{(XI)}\\ 2\alpha + 2\beta &= 0.3724 && \text{(XII)}\\ 2n + 2\beta &= 1.3180 && \text{(XIII)}\\ 4\alpha + 2\gamma &= 0.6020 && \text{(XIV)} \end{aligned}$$

The solution is: $n = 0.6190$; $\alpha = 0.1462$; $\beta = 0.0400$; $\gamma = 0.0086$

The experimental dipole moment of pyrrole is 1.80 $D\uparrow$. Its estimated σ-moment[6] is ~ 0.40 $D\uparrow$. Therefore, a π-moment near 1.40 $D\uparrow$ must be expected. We calculate $(0.2924 \times 0.8000 + 0.0800 \times 2.110) \times 4.802 = 1.93$ $D\uparrow$, in reasonable good agreement with the expected value.

Atomic and bond charges are distributed as shown in Fig. 5.

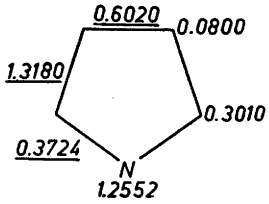

Fig. 5. Atomic and bond charges in pyrrole (model in Table 1). Usual symbolism.

In the following section the chemical properties will be discussed.

RELATION BETWEEN π-ELECTRON DISTRIBUTION AND CHEMICAL PROPERTIES

Neither the existing theoretical background, nor the experimental evidence presented in the literature up till now permit that a rigorous discussion of this topic can be carried through. In the first place it seems certain that chemical reaction rates cannot be debated exhaustively solely by speaking of π-electron distributions. This is too often neglected in papers dealing with molecular orbital treatment of aromatic molecules. The σ-moment must also play a rôle. Secondly, this might be a minor objection as compared with the fact that the true "reaction formulae" of the partners reacting are generally more or less obscure. The following statements, therefore, only hold true to the (unknown!) extent to which all other factors of importance can be ignored.

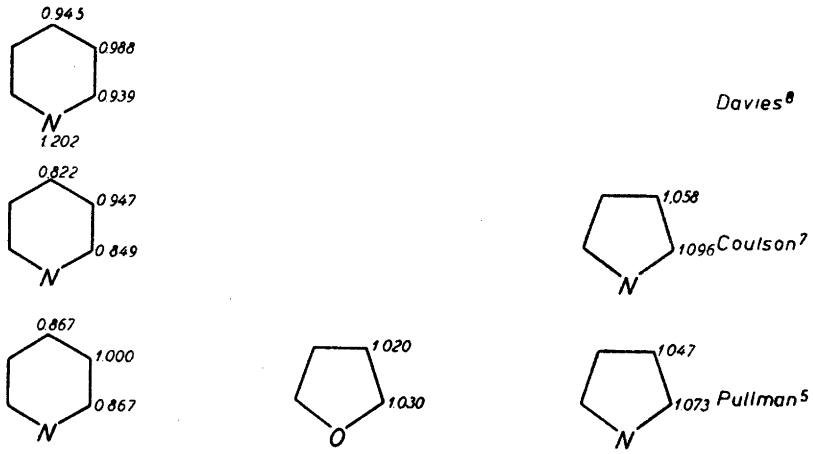

Fig. 6. Molecular diagrams found by molecular orbital treatment (Coulson and Davies) and valence-bond method (Pullman).

In the molecular orbital treatment of molecules[7] a molecular orbital is given by

$$\psi = \sum_1^n c_i \psi_i \qquad \sum_1^n c_i^2 = 1$$

where φ_i are *atomic* orbitals (L.C.A.O.method). For the molecules here considered n = 6. The six orbitals of lowest energy may be found and by summing, say, c_4^2 over all six orbitals a number is obtained which has been called the *total electronic charge* on nucleus 4. The results obtained by this procedure may be presented diagrammatically as shown in Fig. 6.

The electrons should not be thought of as almost entirely on the nuclei, but the numbers indicate the fraction of electron charge which is located at a certain atom *and* extending in the bond directions. Similar numerical data from the present investigation are obtained if the atomic charge in Figs. 2, 4, and 5 are added to *half* the charge in the adjacent bonds (not including the C,H bond). This procedure gives the diagrams of Fig. 7.

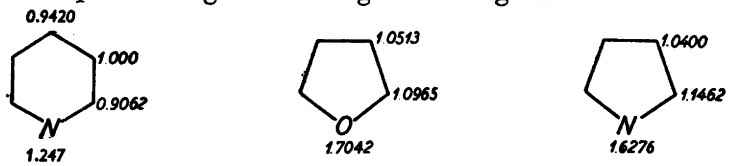

Fig. 7. Molecular diagrams from this investigation.

Taken in the usual way all diagrams of Figs. 6 and 7 equally well "explain" why pyridine prefers *electrophilic substitution, e.g.* bromination at carbon in the 3-position, and furan and pyrrole very pronouncedly react in the 2-position where the greatest electronic density is found. *Nucleophilic substitution* (*e. g.* amination) will be expected to take place in regions of *low* electron density. For pyridine, Pullman's and Coulson's models indicate almost equal probability for substitution in the 2- and the 4-positions in contrast to experiments which show that *e. g.*, amination of pyridine occurs in the 2-position. This fact is somewhat more satisfactorily predicted by the diagram resulting from the present investigation.

A comparison between the calculated and "experimental" molecular diagrams is, however, not quite straightforward because the diagrams of Fig. 6 are based on molecular models which are now definitely known to be wrong (*e. g.* pyridine as a regular hexagon). A recalculation is intended using molecular orbital theory on more correct models.

Table 2 summarizes the results obtained.

Table 2. Weights of resonance components (in per cent) for pyridine, furan and pyrrole. Calculated and measured dipole moments (in Debye units).

	Pyridine	Furan	Pyrrole
"Classical" structure	62 %	65 %	62 %
α-ionic structures	19 %	19 %	29 %
β-ionic structure	—	10 %	8 %
γ-ionic structures	5 %	—	—
'Dewar' structures	14 %	6 %	1 %
Calculated dipole moment	2.26	0.51	2.33
Experimental —•—	2.26	0.66	1.80

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Comparative Crystal Field Studies of some Ligands and the Lowest Singlet State of Paramagnetic Nickel(II) Complexes

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The spectra of the ethylenediaminetetraacetates and some other complexes of the first transition group are compared to the spectra of aquo ions. The position of acetylacetonate in the spectrochemical series is found, and the nickel complexes seem to be octahedral. The influence of tetragonal crystal fields in copper(II) complexes is found by comparison with the analogous nickel(II) complexes. The tris-complexes of α,α'-dipyridyl and *o*-phenanthroline with both metals have the absorption spectra predicted by the crystal field theory of a cubic complex. In a series of nickel(II) complexes, the lowest singlet state $^1\Gamma_3(D)$ is shown to intermix strongly with the triplet states, giving rise to double bands in several cases. The energy decrease of this state at increasing crystal field strength must be due to interactions with some states of other electron configurations.

The absorption band of nickel(II) hexaaquo ion in the red is double as pointed out by J. Bjerrum [1], and can be analyzed into two Gaussian curves [2]. But when the crystal field calculations * of Ilse and Hartmann [3] or Santen and Wieringen [4] are applied [5] to $Ni(H_2O)_6^{++}$, one is led to expect the appearance of only one band, corresponding to the transition from the groundstate $^3\Gamma_2(F)$ to $^3\Gamma_4(F)$. Ballhausen [6] explained the splitting as either due to tetragonal effects, which seems highly improbable [7], or to (L,S) coupling effects. If the latter are only connected with the levels [8] in the free ion $^3F_4$, $^3F_3$, $^3F_2$ and $^3P_2$, $^3P_1$, $^3P_0$, it is not easily explained why the third band due to $^3\Gamma_4(P)$ does not show similar splitting, and why only the hexaaquo ion and a few other complexes [9, 10] show the band $\sim$700mμ double. The present author maintains [11] that the singlet level $^1D_2$ is intermixed with the triplet levels due to (L,S) coupling effects. This level splits in the crystal field [3] of an octahedral complex to $^1\Gamma_3$ of lowest energy and $^1\Gamma_5$ of highest. Later [12], some of

* Recently, Tanabe and Sugano [61] made a very extensive study of the energy levels in octahedral complexes. These authors calculated all the energy matrices of d^n-configurations in crystal fields of cubic symmetry by Racah's methods. The effects of (L,S)-coupling and of interaction with other electron configurations were not investigated.

the experimental results from this paper were used in a demonstration of the change of the relative positions of ${}^3\Gamma_5(F)$ and ${}^3\Gamma_4(F)$ and ${}^1\Gamma_3(D)$ by increasing crystal fields. This behaviour will be discussed below in the theoretical section. For calibration of the crystal field strength scale, it will be useful to compare the absorption spectrum of a given nickel(II) complex with those of other first transition group complexes with the same ligand. In this way, the position of the ligand can be found in the spectrochemical series of hypsochromy, originated by Fajans [13] and extended by Tsuchida [14]. The next three sections involve ethylenediaminetetraacetates and glycinates, aromatic diamines, and acetylacetonates of different transition group ions. In the case of the acetylacetonate comparison with other metals is necessary to demonstrate that the nickel(II) complex is six-coordinated.

ETHYLENEDIAMINETETRAACETATES AND GLYCINATES

The transition group complexes of $[-CH_2N(CH_2COOH)_2]_2$ (here denoted H_4 enta) were first prepared by Brintzinger, Thiele and Müller [15] and later extensively investigated by Schwarzenbach *et al.*[16-19] The latter authors demonstrated the exclusive formation of complexes between one metal ion and one enta^{-4}. In the case of chromium(III), the two nitrogens and three of the four carboxyl groups are coordinated on five places, leaving a water molecule on the sixth. Thus, the free carboxyl group can take up a proton

$$Cr(H_2O)enta^- + H^+ \rightleftharpoons Cr(H_2O)enta\ H \qquad (1)$$

The two forms have the same absorption spectrum, as predicted by crystal field theory where the perturbations from the ligands decreases with about sixth power of the distance. But with pK = 7, the complex gives a proton off:

$$Cr(H_2O)enta^- \rightleftharpoons Cr(OH)enta^{--} + H^+ \qquad (2)$$

and the environment of the chromium(III) ion is changed profoundly with resulting change in spectrum. These spectra have earlier been measured by Hamm [20] and it is seen of Table 1 that the absorption bands have slightly higher wavenumbers than those of the aquo ion. Table 1 gives the band maxima of the ethylenediaminetetraacetate and aquo complexes of the first transition group, except those of nickel and copper, which can be found in Tables 2 and 4. Fig. 1 gives the observed absorption spectra of several of the ethylenediaminetetraacetate complexes.

In the case of cobalt(III), Schwarzenbach found a rather unstable $Co(H_2O)enta^-$, which in alcaline solution changes colour, due to formation of $Co(OH)enta^{--}$ (see eq. 2), and a more stable Co enta$^-$, which does not exhibit such changes. In the latter complex, all six places are used by enta^{-4}. It is seen of Table 1 that the wavenumbers are somewhat larger than in the aquo ion, which according to C. E. Schäffer has maxima at 16 600 and 24 900 cm^{-1}. The nearly identical spectra of, *e.g.* $Cr(OH)enta^{--}$ and $Co(OH)enta^{--}$ do not prove[7] that the first band is due to states with the same quantum number, *viz.* Γ_5, in both cases. In other ethylenediaminetetraacetate complexes the configuration is not exactly known, but one carboxyl group seems to take

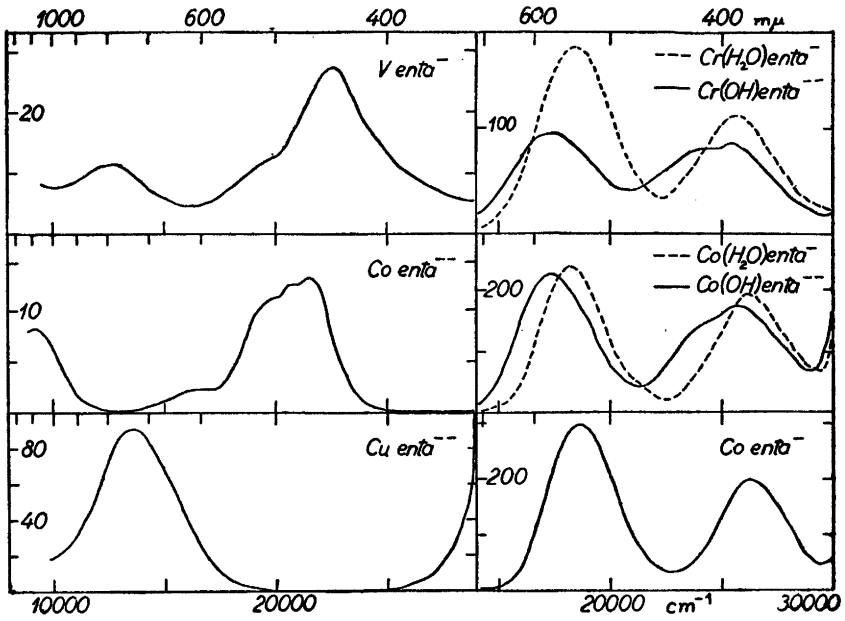

Fig. 1. Absorption spectra of ethylenediaminetetraacetates.
0.08 M V enta⁻, pH∼8.
0.02 M Cr(H₂O) enta⁻ (pH∼4) and Cr(OH) enta⁻⁻ (pH∼10).
0.1 M Co enta⁻⁻, pH∼8.
0.01 M Co(H₂O) enta⁻ (pH∼2) and Co(OH) enta⁻ (pH∼10).
0.01 M Cu enta⁻⁻, pH∼8.
0.01 M Co enta⁻, pH∼2.

up [19] protons. Thus, $M(H_2O)$enta is most likely. The crystal field of such an entity would have a cubic part [7] determined by two amino-, three carboxyl and one water positions. Since the carboxyl group is placed before water in the spectrochemical series and the amino group much later than water, a position of enta^{-4} slightly after H_2O might be predicted. But the complexes will show deviations from the cubic crystal field which will result in splitting of the bands [7]. Holleck and Eckardt [21] have used enta^{-4} as ligand for lanthanide ions and removed nearly all the $(2J+1)$-fold degeneracy of a given level, because the crystal field has so low a symmetry. The complex with divalent cobalt shows three bands at the position predicted [22] for $^4\Gamma_4(P)$, where the aquo ion also exhibits fine structure [2]. Here, the mixture of (L,S) coupling effects and deviations from cubic symmetry cannot easily be disentangled. Even with the two nitrogens in *cis*-position, there exist two possible geometrical isomers of $M(H_2O)$enta with rhombic and tetragonal symmetry [7], respectively. The two forms have the water in *trans*- or in *cis*-position to nitrogen.

As will be shown below, the nickel(II) complex has an absorption spectrum mainly of "cubic" appearance and with no splittings except the small band, due to $^1\Gamma_3$. The nickel(II) complexes of other amino-acids show a similar beha-

Table 1. Absorption band maxima for ethylenediaminetetraacetate and aquo complexes of the first transition group (except of Ni++ and Cu++). Wavenumbers in cm^{-1}. Shoulders in parentheses.

Ti $(H_2O)_6^{+++}$	20 300
Ti enta$^-$	18 400
V $(H_2O)_6^{+++}$	17 700; 25 600
V enta$^-$	12 500; (19 400); 22 600
VO^{++}	12 900; (15 400)
VO enta^{--}	12 800; 17 200
Cr $(H_2O)_6^{+++}$	17 400; 24 700
Cr enta H_2O^-	18 400; 25 600
Cr enta OH^{--}	17 300; (23 800); 25 300
Fe $(H_2O)_6^{++}$	10 400
Fe enta^{--}	9 700
Co $(H_2O)_6^{++}$	8 100; (16 000); 19 400; (21 550)
Co enta^{--}	9 100; (16 300); 19 900; 20 600; 21 500
Co $(H_2O)_6^{+++}$	16 600; 24 900
Co enta$^-$	18 650; 26 300
Co enta H_2O^-	18 200; 26 200
Co enta OH^{--}	17 400; (24 100); 25 900
Co enta Br^{--}	17 100; 25 500

viour in absorption spectrum. Thus, a tris(glycinato) complex * can be identified in the solutions with 0.1 M $Ni(NO_3)_2$ and between 0.3 and 0.5 M potassium glycinate, here denoted K gly. As expected from the crystal field theory, Ni enta^{--} and Ni gly$_3^-$ have band maxima at nearly the same places. The intensity of the first band of Ni gly$_3^-$ is not so anomalously high as that of Ni enta^{--} (see Table 3). Similar behaviour is found in chromium(III) glycinates, compared to Cr enta$^-$, where the first band is due to the transition Γ_2—Γ_5 analogous to the first band of paramagnetic nickel(II) complexes.

Copper(II) bis (glycinate) has the band at a much higher wavenumber than Cu enta^{--}. (See Table 2.) As will be discussed in the next section, Cu gly$_2$ must have a crystal field of pronounced tetragonal symmetry [23], while Cu enta^{--} is less tetragonal, and the tris-complexes of aromatic diamines are nearly cubic with correspondingly low wavenumbers of their band maxima.

At the beginning of the first transition group, the absorption spectra of enta-complexes show some peculiarities. The titanium(III) complex, which easily reduces water with evolution of hydrogen, has the maximum at 18 500 cm^{-1}, while that of Ti $(H_2O)_6^{+++}$ is situated [24] at 20 300 cm^{-1}. This smaller crystal field than water is also found in the yellow V enta$^-$ of trivalent vanadium [18]. While the tris-complexes of dicarboxy-acids, according to Bürger [25], have two bands at smaller wavenumbers than those of V $(H_2O)_6^{+++}$, the ethylenediaminetetraacetate has a quite complicated spectrum (Table 1). It might support the chemical evidence ** that amino groups seem to be weaker bound to V^{+++} than to Cr^{+++} and the heavier ions. In a following paper, the spectra of vanadium(IV) complexes will be discussed.

* Flood and Lorås [50] have determined the three consecutive formation constants in the nickel(II) glycinate system, log K_1 = 5.77, log K_2 = 4.80, and log K_3 = 3.61. In the corresponding copper(II) system, log K_1 = 8.22, and log K_2 = 6.97, while log K_3 is very small. From the spectra, the latter constant can be estimated: log $K_3 \sim 0.36$ by Keefer.[58]

** if the configuration of the complex is not so different from the octahedral that a diamagnetic groundstate is stabilized (*cf.* $Mo(CN)_8^{-4}$ investigated by Griffiths, Owen and Ward [53]).

α,α'-DIPYRIDYL- AND *o*-PHENANTHROLINE COMPLEXES

Roberts and Fields [9] and Basolo, Hayes and Neumann [26] have measured the spectrum of Ni $phen_3^{++}$ which exhibits bands at slightly higher wavenumbers than Ni en_3^{++}, corresponding to a larger value of the crystal field strength (E_1—E_2). The interesting fine structure of the first band will be discussed below. In the present section, the copper(II) complexes will be compared to those of nickel(II). The maximum coordination number [1] 6 of copper(II) is easily obtained with three molecules of α,α'-dipyridyl and *o*-phenanthroline, in contrast to the behaviour of the ligands ammonia and ethylenediamine. While the absorption spectra in the latter cases show "pentammine effects" [23], *i.e.* the band maxima are displaced towards lower wavenumbers by the uptake of the fifth NH_3 or third en, the spectra of the complexes of aromatic diamines with divalent copper have a regular cubic appearance. Thus, Cu dip_2^{++} and Cu $phen_2^{++}$ have their broad maxima at a lower wavenumber than the tris-complexes. Fig. 2 shows the spectra of the two

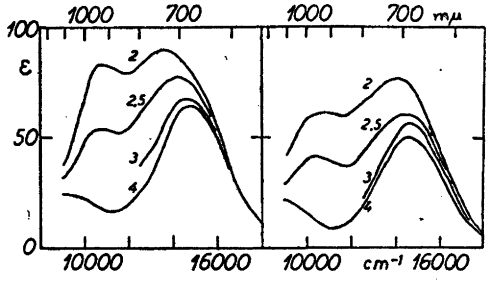

Fig. 2. Absorption spectra of copper (II) complexes of aromatic diamines.

Left-hand side a,a'-dipyridyl, right-hand side o-phenanthroline. Moles of diamine per Cu++ given as numbers of curves (2, 2.5, 3, 4). Curve 4 represents the pure tris-complex. The solutions contain about 50 % ethanol and 0.02 M Cu.

systems with 2, 2.5, 3 and 4 molecules of diamine added per copper ion. The high intensity of the bis-complex, which can be extrapolated from the figure, supports the idea that is has mainly the *cis*-configuration. The *trans*-configuration would have a less intense band at a higher wavenumber than the tris-complex in analogy to Cu en_2^{++}. The weak bands found below 9 000 cm^{-1} in Cu $phen_3^{++}$ and Cu dip_3^{++} are not easily explained from the crystal field theory.*

* Hartmann [39], Owen [46], and Orgel [45] have elaborated the old suggestion by Van Vleck [52] that the intermixing of molecular γ_3-orbitals would produce the same effect as the electrostatic crystal field separation of γ_3 and γ_5 of 3d-electrons, i.e. an increase of (E_1—E_2). In tetragonal complexes with four 3d-levels, the molecular orbital intermixing may produce much more individual behaviour of the levels than suggested by the calculations on pure $3d^n$-configurations [7, 46]. Much evidence can be found (also from diamagnetic, planar nickel (II) complexes) that γ_{t_2} has unusually high energy. Thus, the band $^2\Gamma_{t_2} \rightarrow {}^2\Gamma_{t_1}$ may be situated $\sim$ 8 000 cm^{-1} in nearly cubic copper (II) complexes and $\sim$ 10 000 cm^{-1} in the more tetragonal hexaaquo ion [23], while it is masked by the principal band in the most tetragonal complex Cu en_2^{++}.

Table 2. The first absorption band of several nickel(II) and copper(II) complexes. Double bands are given in parentheses. The ratio between the wavenumbers is given to show tetragonality effects.

Complex	ν_{Ni}	ν_{Cu}	ν_{Cu}/ν_{Ni}
$(H_2O)_6$	8 500	12 600	1.48
$(NH_3)_4(H_2O)_2$	~10 000	16 900	1.7
$(NH_3)_6$	10 750	15 600	1.45
$en_2(H_2O)_2$	(10 000; 11 100)[6]	18 200	1.72
en_3	11 200	16 400	1.46
$gly_2(H_2O)_2$	9 800	15 800	1.60
gly_2	10 100	~15 100	1.50
enta H_2O	10 100	13 700	1.36
$dip_2(H_2O)_2$	—	(10 500; 13 900)	1.1
dip_3	(11 500; 12 650)	14 700	1.21
$phen_2(H_2O)_2$	10 900[26]	(10 200; 13 300)	1.15
$phen_3$	(11 550; 12 700)	14 700	1.21

Table 2 gives the wavenumbers of the first band maximum of a series of nickel(II) and copper(II) complexes. If the complexes were cubic, the wavenumbers would in both cases [27] be equal to $(E_1—E_2)$. This quantity would be expected [5] to be of the same magnitude in divalent nickel and copper. The ratio between the observed wavenumbers ν_{Cu}/ν_{Ni} gives a relative measure for the tetragonality of the copper complex, because the nickel(II) spectra are nearly not affected by tetragonality. It is seen of Table 2 that ν_{Cu}/ν_{Ni} varies from 1.1 in cases where the copper complexes approximates cubic symmetry to 1.7 in the cases of strong tetragonality, as *e.g.* the bis(ethylenediamine) complex.

Table 3. The absorption bands of acetylacetonates and other oxo complexes of the first transition group. Wavenumbers in cm^{-1}. Shoulders in parentheses.

Cr $(H_2O)_6^{+++}$	17 400; 24 700
Cr ox_3^{---}	17 400; 24 000
Cr aca_3	17 900; (22 800; 24 400); 25 800; 26 500
Co $(H_2O)_6^{+++}$	16 600; 24 900
Co ox_3^{---}	16 500; 23 800
Co $(CO_3)_3^{---}$	15 700; 22 800
Co aca_3	16 900, —
Ni $(H_2O)_6^{++}$	8 500; 13 500; (15 400); 25 300
Ni aca_2	8 800; (12 900); 15 250, —
Ni $aca_2(C_2H_5OH)_2$	9 100; (13 000); 15 550, —

ACETYLACETONATES

2,4-Pentanedione (here denoted H aca) forms complexes with metals, which usually are neutral molecules, *i.e.* inner-salts. The place of aca^- in the spectrochemical series can be found from the chromium(III) and diamagnetic cobalt(III) complexes, which certainly are cubic [11]. It is seen of Table 3 that in both cases the wavenumbers are slightly larger than for the hexaaquo ions. In the ultraviolet a strong electron transfer spectrum appears, which masks the second band of the green Co aca_3. Other green cobalt(III) complexes have

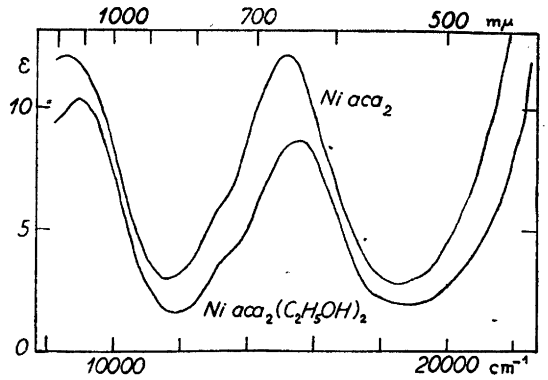

Fig. 3. Absorption spectra of 0.05 M anhydrous nickel (II) bis (acetylacetonate) in benzene and of the same solution with 2 volume % ethanol added.

smaller (E_1—E_2) than H_2O, such as the tris(carbonato) complex given in Table 3.* The spectra of tris(oxalato) complexes were measured by Mead [28].

These examples show that the concept of the Orgel diagram [5, 29] has only an approximative character, when very fine details are considered. This diagram gives the energy levels as function of only one cubic crystal field strength (E_1—E_2). Thus there should exist a treshold value of (E_1—E_2) of diamagnetism in cobalt(III) complexes, which is not reached in the hexafluoro complex, while it is exceeded in the tris(carbonato) complex. This seems to contradict the usual order of CO_3^{--} and F^- in the spectrochemical series. The tris(oxalato) complexes of trivalent chromium and cobalt have a smaller spacing between the two strong bands than have the aquo ions. This departure from the regular evolution of levels in the Orgel diagram might be ascribed to interactions with other electron configurations [12]. In this special case, the intermixing is presumably due to electron transfer states, which seem to have a great influence on the intensities of the absorption bands of oxalato and thiocyanato complexes [11].

The anhydrous form of Ni aca_2 is soluble with dark green colour in benzene or carbon tetrachloride. If water is added, the dihydrate precipitates out. The latter complex has undoubtedly octahedral configuration, as found by Kanekar [30, p. 404]. If ethanol is added in small quantities (not much more than 2 moles per nickel atom) to the dark green solutions, they turn bright bluish green with a displacement of their bands towards higher wavenumbers. The latter form is presumably the octahedral Ni $(aca)_2$ $(C_2H_5OH)_2$. Besides the steep absorption in the blue, due to the electron transfer band, the two

* Shimura, Ito and Tsuchida [54] have studied several oxo complexes, *e.g.* the heteropolymolybdates with six oxygen atoms around the metal ion, as also studied by Baker *et al.* [55]. The absorption bands are situated [54] at 18 400 and 25 000 cm^{-1} of Cr^{+3} and at 16 500 and 24 300 cm^{-1} of Co^{+3} with lower ε than the aquo ions. For Mn^{+4}, a broad band is observed [54] at 21 400 cm^{-1}, while the narrow spin-forbidden d^3-band $^4\Gamma_2$—$^2\Gamma_3$ is found at 14 300 cm^{-1}. In the ruby, the present author has measured the spin-allowed bands at 18 500 and 24 400 cm^{-1}.

forms each show two bands and shoulder on the second band, as given in Table 3 and shown in Fig. 3.

In Ni $(aca)_2(C_2H_5OH)_2$, these two bands correspond undoubtedly to the transitions from ${}^3\Gamma_2(F)$ to ${}^3\Gamma_5(F)$ and ${}^3\Gamma_4(F)$, because the crystal field is of similar strength to that of the aquo ion. This is further supported by the sky-blue colour of the ammonia addition compound Ni $(aca)_2(NH_3)_2$, where the cubic contribution [7] is larger.

The anhydrous Ni aca_2 seems now also to be mainly octahedral, perhaps with solvate molecules perpendicular to the plane of the two acetylacetonate ligands. The relatively low intensity of the crystal field bands does not either support the tetrahedral configuration.*

NICKEL(II) COMPLEXES

In Table 4, the observed bands of a series of nickel(II) complexes are given with their parameters [2]: wavelength λ_n, wavenumber ν_n and molar extinction coefficient ε_n, all in the band maximum, and the half-widths δ (—) and δ (+) towards smaller and larger wavenumbers. In the case of $Ni(H_2O)_6^{++}$, a Gaussian analysis was performed [2], while the other entries in the table refer to directly observed maxima. Further, the approximate value of the oscillator strength P is given by [2]:

$$P = 4.60 \times 10^{-9} \varepsilon_n [\delta\,(-) + \delta\,(+)] \qquad (3)$$

In relation to Ballhausens's paper on intensities [31] the ratio PR/ν_n is also given, which in some cases is assumed to be approximately constant [31]. R is the Rydberg constant 109 740 cm^{-1}. Fig. 4 gives the observed spectra in the wavenumber range 8—17 000 cm^{-1}, where the ${}^1\Gamma_3$ phenomenae discussed below take place. The following complexes are arranged according to increasing value of $(E_1—E_2)$:

Sulphuric acid solutions. The yellow solution of nickel(II) carbonate in concentrated sulphuric acid presents the lowest $(E_1—E_2)$ observed. The stoichiometric composition of the complexes is not known, but the solution is rather unchanged by addition of even 20 % of water.

Phosphoric acid solutions. The yellowish-green solutions of nickel(II) carbonate in 85 % phosphoric acid are very sensitive to temperature. The smaller $(E_1—E_2)$ developed in the warm, yellow solutions can partly be ascribed to bathochromic effects, common to all complexes [22] and partly to formation of complexes with polyphosphate ligands.

Thiocyanate solutions. Mr. K. G. Poulsen has kindly informed me that in strong thiocyanate solutions, the intensity ratio between the two parts of the band in the red is reversed, compared to the aquo ion. It has been meas-

* Recently, Hartmann and Fischer-Wasels [56] published extensive calculations on the energy levels of tetrahedral and tetragonal nickel (II) complexes. Since the tris (diamine) complexes presumably are octahedral, and since *e.g.* the aquo ion exhibits smooth changes in spectrum by uptake of six ammonia molecules [1], these simple nickel (II) complexes are not tetrahedral, and it is still in doubt, if any exists. As will be discussed in another paper, it is also improbable that the manganese (II) aquo ion is tetrahedral, as maintained by Schläfer [57]. The only evidence for tetrahedral configuration of divalent $3d^n$-metal ions in solution is the zinc tetrammine ion [1] and the cobalt halide complexes [22, 45].

Table 4. Absorption Bands of Nickel(II) Complexes in Solution.

Complex and identifications of the excited states:	λ_n mμ	ν_n cm^{-1}	ε_n	$\delta(-)$ cm^{-1}	$\delta(+)$ cm^{-1}	P × 10^{-5}	PR/ν_n × 10^{-5}
Sulphuric acid ((E_1-E_2) = 7 000 cm^{-1}):							
$^3\Gamma_4(F)$	820	12 200	2.6	~1 200	~1 000	2.6	23
$^1\Gamma_3(D)$	680	14 700	0.5	—	400	0.2	2
$^3\Gamma_4(P)$	428	23 350	8.3	1 400	1 400	10.7	50
Phosphoric acid (7 900):							
$^3\Gamma_4(F)$	760	13 150	3.2	1 400	1 500	5.3	40
$^1\Gamma_3(D)$	672	14 900	1.8	—	500		
$^3\Gamma_4(P)$	408	24 500	8.5	1 600	1 400	11.8	53
Hexaaquo ion (8 500):							
$^3\Gamma_5(F)$	1 180[35]	8 500	2.0	1 200	1 200	2.2	28
$^3\Gamma_4(F)$	740 [a]	13 500	1.8	1 100	1 100	3.5	27
$^1\Gamma_3(D)$	650 [a]	15 400	1.5	1 200	1 200		
$^3\Gamma_4(P)$	395 [a]	25 300	5.2	1 500	1 500	7.2	31
Thiocyanates (8 600):							
$^1\Gamma_3(D)$	725	13 800	5	~1 000	—	12	80
$^3\Gamma_4(F)$	655	15 300	7	—	~1 300		
$^3\Gamma_4(P)$	393	25 500	16	1 600	1 600	23	100
Ethylenediaminetetraacetate (10 000):*							
$^3\Gamma_5(F)$	990	10 100	31	1 400	1 300	38	420
$^1\Gamma_3(D)$	790	12 650	5	—	500	2.3	20
$^3\Gamma_4(F)$	587	17 000	8.3	1 700	1 550	12.4	80
$^3\Gamma_4(P)$	382	26 200	12.2	1 750	2 300	22.7	95
Tris(glycinate) (10 100):							
$^3\Gamma_5(F)$	990	10 100	9.9	—	1 500	13.6	145
$^1\Gamma_3(D)$	763	13 100	2.0	—	300	0.6	5
$^3\Gamma_4(F)$	602	16 600	8.2	1 800	1 800	13.6	90
$^3\Gamma_4(P)$	362	27 600	14.4	1 700	1 800	23.2	92
Hexa(ammonia) (10 800):							
$^3\Gamma_5(F)$	930	10 750	4.0	1 500	1 400	5.3	54
$^1\Gamma_3(D)$	760	13 150	0.5	—	~500	0.2	2
$^3\Gamma_4(F)$	571	17 500	4.8	1 550	1 600	7.0	44
$^3\Gamma_4(P)$	355	28 200	6.3	1 600	1 800	9.8	38
Tris(ethylenediamine) (11 600):							
$^3\Gamma_5(F)$	890 [a]	11 200	7.3	1 250	—	10.3	98
$^1\Gamma_3(D)$	805	12 400	5	—	—		
$^3\Gamma_4(F)$	545 [a]	18 350	6.7	1 650	1 650	10 2	61
$^3\Gamma_4(P)$	345 [a]	29 000	8.6	1 700	1 900	14.3	54
Tris(α,α′-dipyridyl) (12 100):							
$^1\Gamma_3(D)$	868	11 500	5.7	850	—	9.6	86
$^3\Gamma_5(F)$	790	12 650	7.1	—	1 250		
$^3\Gamma_4(F)$	521	19 200	11.6	1 700	2 100	20	~110
Tris(*o*-phenanthroline) (12 200):							
$^1\Gamma_3(D)$	865	11 550	5.5	1 000	—	9.4	84
$^3\Gamma_5(F)$	788	12 700	6.8	—	1 350		
$^3\Gamma_4(F)$	519	19 300	11.9	1 650	1 700	19	~110

* Hamm [20] observed an intermediate form of $Cr(H_2O)$ enta$^-$, perhaps another geometrical isomer. There is some evidence that Ni enta^{--} for some minutes can exist with the absorption maxima at 9 900, 12 900, 16 900, and 26 400 cm^{-1} with ε_n = 25, 2, 8 and 14, respectively.

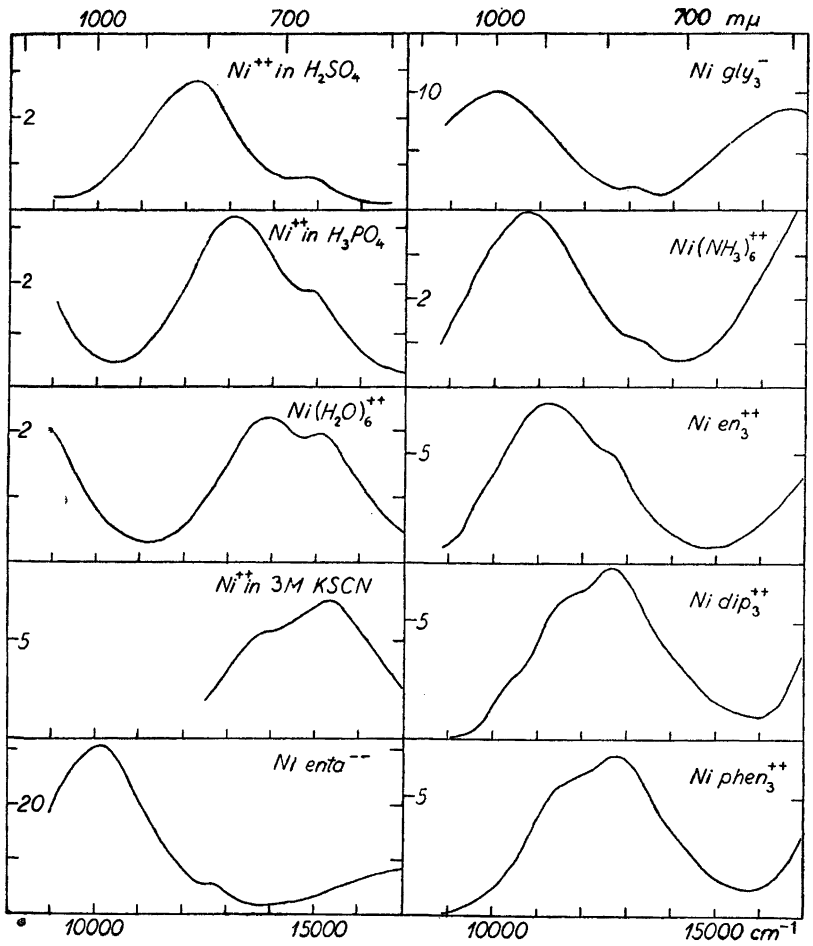

Fig. 4. Absorption spectra of nickel(II) complexes in the range 8 000—17 000 cm⁻¹.

0.02 M Ni in 98 % H_2SO_4 — *0.1 M $Nigly_3^-$ (from 0.4 M Kgly).*
0.1 M Ni in 85 % H_3PO_4 — *0.2 M Ni $(NH_3)_6^{++}$ in 10 M NH_3.*
0.5 M Ni $(NO_3)_2$ in H_2O — *0.2 M Ni en_3^{++} in 1 M en*
0.1 M Ni in 3M KSCN — *0.02 M Ni dip_3^{++} (from 0.08 M dip, 80 % ethanol)*
0.05 M Ni $enta^{--}$, pH∼8. — *0.02M Ni $phen_3^{++}$ (from 0.08M phen, 80 % ethanol)*

ured by Fronæus [32] and is seen in Fig. 4 for a solution, 3 M in KSCN, which probably [32] contains complexes with at least 3 thiocyanates per Ni. Since $^3\Gamma_4(P)$ has nearly the same position as in the aquo ion, the crystal field strength is not much larger.

Hexa(ammonia) and tris(ethylenediamine) complexes. Their absorption spectra are quite similar with about 7 % difference in $(E_1—E_2)$ The shoulder at 12 400 cm^{-1} in Ni en_3^{++} caused the large value of $\delta(+)$, found by the author [2].

Tris(α,α'dipyridyl) and tris(o-phenanthroline) complexes. The crystal field bands of Ni dip_3^{++} correspond to $(E_1—E_2)$ being only 0.5 % smaller than in Ni $phen_3^{++}$. In the ultraviolet, very strong absorption is observed in both cases, but the α,α'-dipyridyl complex seems to have a smooth increase below 32 000 cm^{-1}, while the *o*-phenanthroline complex exhibits very characteristic, narrow bands [9]. The band $^3\Gamma_2(F)$ —$^3\Gamma_4(P)$ predicted ~30 000 cm^{-1} is totally hidden by these high bands common to all complexes of aromatic diamines [33]. The crystal field bands ~19 300 cm^{-1} might be suspected to be partially due to impurities of iron(II) complexes, which here have [33] $\varepsilon \sim 10\,000$. But attempts to oxidize any trace of iron to the trivalent state before addition of the diamine did not change the spectrum of the nickel(II) complex, and the present author agrees with Roberts and Field [9] in the values of ε_n.

In Table 4, the values of P are calculated from the hypothesis of equal half-widths, if only one is known. In the case of shoulders, only the total sum of P is calculated as rationalized in the theoretical discussion. The first band of $Ni(H_2O)_6^{++}$ is measured by Dreisch and Trommer [34]. The value of $(E_1—E_2)$ given after the name of each complex is taken as the energy difference between the centre of gravity of $^3\Gamma_5(F)$ and the groundstate $^3\Gamma_2(F)$, rather than the values earlier given [5], which were fitted to all three strong bands. The new values are used for the Orgel diagram in Fig. 5.

THEORETICAL

The preceding absorption spectra of nickel(II) complexes give the possibility of drawing the Orgel diagram with exceptionally small differences between the individual $(E_1—E_2)$ of the complexes, and thus to investigate the concept of lines in the Orgel diagram determined as eigenvalues of matrices [7,29] similar to those for intermediate coupling [35]. After the recent investigation by Shenstone [36], the levels of the free ions Ni^{++} are:

[A] $3d^8$:	$^3F_4$	0 cm^{-1}
	$^3F_3$	1 361
	$^3F_2$	2 270
	$^1D_2$	14 032
	$^3P_2$	16 662
	$^3P_1$	16 978
	$^3P_0$	17 231
	$^1G_4$	23 109
[A] $3d^7$ 4s:	^5F	53 704, 54 658, ...
	^3F	61 339, ...
	^5P	71 067, ...
	^3G	75 123, ...
[A] $3d^7$ 4p:	^5F	110 212, ...
	^5D, ^5G, ^3G, ^3F, ...	~112 000

It is seen that the multiplet splitting is rather large, corresponding to a departure from pure Russell-Sounders coupling. In the first transition group, only Cu^{++} with the energy difference 2 072 cm^{-1} between the groundstate

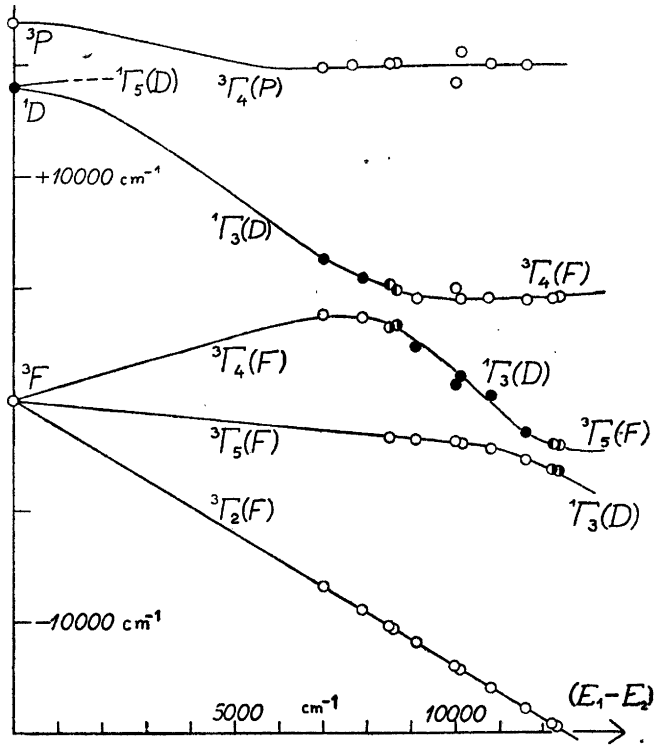

Fig. 5. Orgel diagram of octahedral nickel(II) complexes.
The three triplet levels and the lowest singlet level ($^1\Gamma_3$) as function of the crystal field strength (E_1-E_2). Intermixing effects between the singlet level and the two triplet levels $^3\Gamma_4(F)$ and $^3\Gamma_5(F)$ are shown by the filled circles, which denote singlet character. The values of (E_1-E_2) and positions of the band maxima from Table 4. The groundstates are fixed [27] on the line $-\frac{6}{5}(E_1-E_2)$.

$^2D_{5/2}$ and $^2D_{3/2}$ has a larger dependance on J (the vector sum of L and S, which is given as index for the levels in the free ion). In the theory of intermediate coupling [35], the states with the same J interact as a function of the Landé multiplet splitting factor ζ_{nl} (*cf.* as a concrete example the lanthanide and actinide ions [37]), the pure Russell-Saunders coupling being the limiting case $\zeta_{nl}=0$. In Ni^{++}, ζ_{3d} is calculated to be $\cong 600$ cm^{-1} from the observed levels in the free ion. As seen Ref.[35, p. 269], the interaction between the states with $J=2$ in Ni^{++} is particularly strong, since the non-diagonal elements are

$$(^3F_2 \quad ^1D_2) = -2\sqrt{3/5}\zeta_{3d} \qquad (4)$$
$$(^3P_2 \quad ^1D_2) = \sqrt{21/10}\zeta_{3d}$$

which in both cases have the numerical value ~1 000 cm^{-1}. The usual approximation in crystal field theory, when applied to the transition group ions [38] that the coupling between L and S is negligible compared to the crystal field

strength (E_1—E_2), is thus slightly weakened in the case of nickel(II) complexes, where (E_1—E_2) is ~10 000 cm^{-1}.

The Orgel diagram of nickel(II) complexes, as seen in Fig. 5, seems really to show the interaction between $^1\Gamma_3(D)$ when passing over firstly $^3\Gamma_4(F)$ at (E_1—E_2) = 8 000 cm^{-1} and secondly over $^3\Gamma_5(F)$ at (E_1—E_2) = 12 000 cm^{-1}. These values correspond to the water-thiocyanate and the ethylenediamine —α,α'-dipyridyl range of increasing crystal field strength. If no interaction occurred between $^1\Gamma_3(D)$ and the triplet states, the lines would simply cross in the Orgel diagram without anomalies such as bent curves near the cross-point. But if an interaction occurs with the nondiagonal element [7] of energy = K, the two states will have the minimum distance $2K$ and do not cross each other. Of Fig. 5 can be seen that K is = 800 cm^{-1} between $^1\Gamma_3$ and $^3\Gamma_4(F)$ and = 500 cm^{-1} between $^1\Gamma_3$ and $^3\Gamma_5(F)$, a remarkably large part of the interaction between the pure levels with $J = 2$ which of course cannot be represented by the triplet states in crystal fields.

Contrary to the case of simple crossing (when $K = 0$), the interesting phenomenon of intermixing occurs between the two interacting states. When the distance E_a—E_b between the states decreases, the wavefunctions will be intermixed with coefficients $\cong \left(\frac{K}{E_a—E_b}\right)^2$ in the squares (being 1 %, when $K = 0.1$ (E_a—E_b) *etc.*). The energy of the lowest state will be decreased by the amount $\frac{K^2}{E_a—E_b}$, while the energy of the highest state will be increased by the same amount (this is often called "resonance energy" in chemical textbooks [39]). In the minimum distance, the energy decrease is = K and the intermixing 50 %. After this point, the intermixing as function of the free variable (in the Orgel diagram the crystal field strength) will be reversed; the energy repulsions decrease rapidly and the highest level now assume the wave function, which before the point of minimum distance was characteristic for the lowest level. Actually, at large distance from the point of near crossing, the levels behave as straight lines in the Orgel diagram, and especially as if they *had* crossed. Only near the cross-point of the asymptotes, the intermixing is so large that it has no physical significance to label one of the levels as having just the quantum numbers, characteristic for one of the two asymptotic straight lines.

One of the properties which clearly is intermixed in our case, is the total spin number S. As pointed out by Hellwege [40], the transitions between states with different S is only possible in so far as S is not strictly defined, "is not a good quantum number". This is caused by intermediate coupling effects. The usually cited intensity ratio [5] 0.01 between bands, where S changes 1, and where S is constant, corresponds to an intermixing of 1 % of the other S-value. In the case of nickel(II) complexes, the intermixing of triplet character ($S=1$) in $^1\Gamma_3(D)$ can assume much higher values*, as seen of Table 4,

* The triplet character ($S = 1$) of the weak band is calculated to 0.1 for Ni^{++} in H_2SO_4, 0.1 for Ni^{++} enta, 0.03 for Ni gly_3^- and 0.05 for $Ni(NH_3)_6^{++}$ with the two values of K given above, if the influence of the two triplets is additive. This is in outstanding agreement with the relative values of P, given in Table 4.

where braces in the identification column denote cases, where no distinction can be made between the singlet and the nearest triplet level. Therefore, only the total sum of P is calculated in such cases, because the intensity is distributed proportional to the triplet character of the levels. The question arises as to whether the groundstate ${}^3\Gamma_2(F)$ of paramagnetic nickel(II) complexes would be intermixed with the singlet state when these are crossing, *i.e.* when the magnetism is changed by increasing crystal field strength. If so, this would make this transition point indefinite. But it can be argued that the internal vector group product [7] of Γ_4 (corresponding to $S = 1$) with the other states has the following values:

$$\begin{aligned} \Gamma_2 \times \Gamma_4 &= \Gamma_5 \\ \Gamma_4 \times \Gamma_4 &= \Gamma_1 + \Gamma_3 + \Gamma_4 + \Gamma_5 \\ \Gamma_5 \times \Gamma_4 &= \Gamma_2 + \Gamma_3 + \Gamma_4 + \Gamma_5 \end{aligned} \qquad (5)$$

These sums consist of the quantum numbers of levels with different J[38] from ${}^3F$. Only ${}^3\Gamma_5$ and ${}^3\Gamma_4$ contain representations of Γ_3, which can intermix with ${}^1\Gamma_3(D)$.

Ballhausen [31] has suggested that the deviations from Hellwege's selection rules for octahedral complexes [11, 40] are due to intermixing of the quantum numbers ${}^{2S+1}\Gamma_n$. If this is valid, a similar mechanism must act on ${}^3\Gamma_5(F)$ and distribute it on ${}^3\Gamma_4(F)$ and ${}^3\Gamma_4(P)$ in Ni^{++}, because only the transition Γ_2—Γ_5 is allowed, sofar symmetry reasons are considered [40]. Mr. C. E. Schäffer has pointed out to me by an analogous argument that ${}^4\Gamma_4(F)$, but not ${}^4\Gamma_4(P)$, must be highly intermixed with ${}^4\Gamma_5(F)$ in Cr^{+++}. Another explanation may be found in the coupling with vibrations [39] which produce group products containing several different Γ_n.

Polder [41] discussed the crystal field splittings of ${}^2D$ in Cu^{++} in tetragonal and rhombic crystal fields and pointed out that (L,S) coupling effects can separate the highest level in tetragonal complexes, *viz.* ${}^2\Gamma_{t5}$, into two with the distance ~800 cm^{-1}. Abragam and Pryce [42] investigated the magnetic properties of copper(II) salts along these lines, and Ballhausen [43] later interpreted the absorption spectra on basis of the crystal field model of Ilse and Hartmann [3]. It is interesting that even in a cubic complex of Cu^{++}, the state ${}^2\Gamma_5$ would split due to (L,S) coupling effects in a similar way as in tetragonal fields [43].

Evidently, (L,S) coupling effects have some importance for the absorption spectra of the last elements in the first transition group, *viz.* Co^{++}, Ni^{++} and Cu^{++}. As a further example may be cited the three bands of Co enta^{--} (see Table 1) and fine structure of $Co(H_2O)_6{}^{++}$, discussed by Abragam and Pryce [44]. For a complete analysis of these spectra it is further necessary to consider the interactions with other electron configurations (see the list of levels of Ni^{++} above) which are not very distant in the divalent ions. Besides this, the electron transfer states seem very important in many cases. Especially, Orgel [45] has pointed out that a class of diamagnetic nickel(II) complexes with ligands showing tendency of π-bonding have very peculiar spectra. But also the ordinary complexes, which are only σ-bonding in the theory of Owen [46], have interaction effects. In the present paper is shown that ${}^1\Gamma_3$ is systematically

decreased by increasing $(E_1—E_2)$ in paramagnetic nickel(II) complexes, compared to $^3\Gamma_2$ (see Fig. 5). These two states should, if no electron configuration interaction is assumed, have the asymptotical distance [7]

$$\tfrac{4}{7}(^1D) + \tfrac{3}{7}(^1G) — (^3F) = 18\,000 \text{ cm}^{-1} \quad (6)$$

which is actually found to be 13 000 cm^{-1} or less (see Table 4).

It is seen of Table 4 that $^1\Gamma_3(D)$ generally is situated about 0.45 $(E_1—E_2)$ below the predicted place. Thus, it might be concluded by extrapolation that a purely cubic nickel(II) complex would be diamagnetic, if $(E_1—E_2)>40\,000$ cm^{-1}. But due to the Jahn-Teller effect the state would spontaneously change to a tetragonal configuration [7], and at much lower values of $(E_1—E_2)$, the lowest of the diamagnetic tetragonal levels, $^1\Gamma_{tl}$, will be the groundstate. This is observed in the penta- (and presumably also the hexa-) cyanide complex of nickel(II), which is red-orange with the band of the yellow $Ni(CN)_4^{--}$ displaced towards lower wavenumbers, analogous to [47] $PdCl_6^{-4}$ compared to $PdCl_4^{--}$.

EXPERIMENTAL

Materials. The ethylenediaminetetraacetates in solutions were prepared from stock solutions of chromium(III) nitrate, cobalt(II) sulphate, nickel(II) nitrate and copper(II) sulphate of AnalaR grade and 1 M Na_4enta from analytical pure H_4enta and two moles of Na_2CO_3. At room temperature the complete formation of Ni enta^{--} takes several hours. Co enta^{--} in NaOH was oxidized by H_2O_2 according to Schwarzenbach [17]. Vanadium(III) was prepared from ammonium metavanadate with hydrochloric acid and zinc or from $NH_4V(SO_4)_2$, 12 H_2O after Palmer [48]. Solutions of glycinates were made from 1 M potassium glycinate and the metallic salts. Acetylacetonates were prepared from acetylacetone and stoichiometric amounts of Na_2CO_3 and metallic salts. Ni $aca_2(H_2O)_2$ was made anhydrous by azeotropic distillation with toluene. Co aca_2 was prepared from $Co(OH)_2$ according to Urbain and Debierne [49]. 0.1 M solutions of α,α'-dipyridyl and o-phenanthroline (Merck's p.a.) in ethanol were added to nickel and copper salts.

Measurements. In the wavelength range below 800 $m\mu$, the spectra were measured on a Cary spectrophotometer and recorded at least twice with rather high dispersion. Above 700 $m\mu$ several measurements were performed on the Beckman DU spectrophotometer, and the bands in the infra-red determined with an interval of 10 $m\mu$. In both cases 1 cm, 2 cm, and 5 cm cells were used.

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On the MoO_2 Structure Type

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The crystal structure of vanadium dioxide may be considered as representing the idealized form of the MoO_2 structure type, which also comprises dioxides of molybdenum, wolfram, technetium, and rhenium. The structure is of a deformed rutile type, the metal atoms occurring in doublets. The bond distances are correlated to the number of valence electrons of the metal atoms.

The crystal structures of the isomorphous dioxides of molybdenum and wolfram may be described as being of a deformed rutile type [1]. Thus the metal atoms coordinate six oxygen atoms to form MeO_6 octahedra which are joined by sharing edges to form strings. The strings are mutually connected to a threedimensional structure by octahedra having corners in common. In the ideal rutile structure type, the metal atoms are equidistantly arranged within the strings. In the MoO_2 structure type, however, the metal atoms are alternately nearer to and farther from each other. This results in a pronounced formation of metal atom doublets and distortion of the MeO_6 octahedra. The symmetry is also correspondingly lowered from tetragonal for the rutile type to monoclinic for the MoO_2 structure type. The structures are illustrated in Fig. 1.

The symmetry actually derived for molybdenum and wolfram dioxide is $P2_1$ (No. 4). However, for both compounds only a few weak reflexions $h0l$ (h odd) were observed, which indicates that the symmetry is very close to $P2_1/a$ (No. 14). If the orientation $P2_1/c$ is chosen in order to bring about conformity with the usual notation given in the International Tables for X-Ray Crystallography, the atomic arrangement of molybdenum dioxide is very nearly given by the following table, which is in accordance with the previously published data within the accuracy of the structure determination [1].

MoO_2: Space-group: $P2_1/c$

4 Mo in 4(*e*):	$xyz;\ \bar{x}\bar{y}\bar{z};\ \bar{x},\ \frac{1}{2}+y,\ \frac{1}{2}-z;\ x,\ \frac{1}{2}-y,\ \frac{1}{2}+z$
	$x = 0.232,\ y = 0.000,\ z = 0.017$
4 O_I in 4(*e*):	$x = 0.11,\ y = 0.21,\ z = 0.24$
4 O_{II} in 4(*e*):	$x = 0.39,\ y = 0.70,\ z = 0.30.$

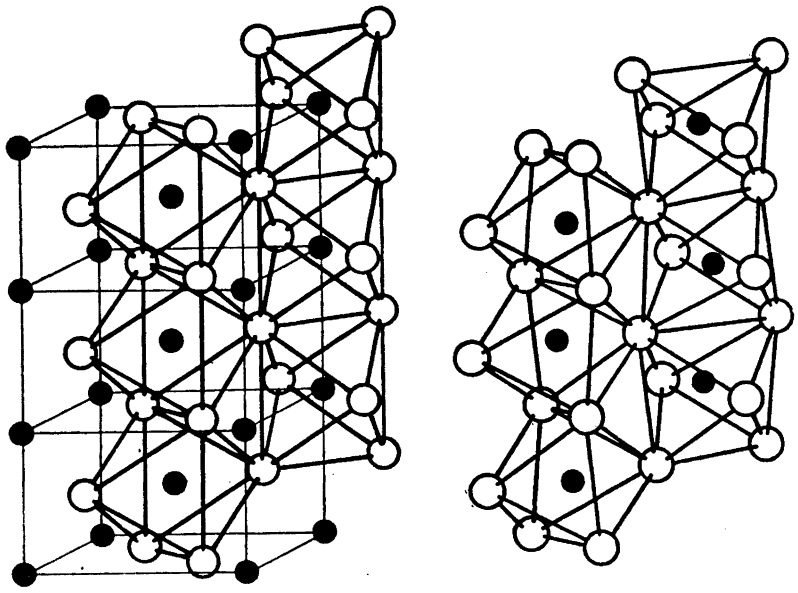

Fig. 1. a) The rutile structure type. MeO_6 octahedra are joined by sharing edges to form strings, which are mutually connected by corners. Three unit cells are indicated.

b) The MoO_2 structure type. The MeO_6 octahedra are joined in the same way as in rutile but the Me atoms within the strings are pairwise drawn nearer to each other to form doublets, which causes distortion of the octahedra.

For titanium dioxide (rutile) an analogous description would give the following set of atomic positions [2,3]:

TiO_2: Space-group: $P2_1/c$

4 Ti in 4(*e*): $x = 0.250$, $y = 0.000$, $z = 0.000$
4 O_I in 4(*e*): $x = 0.097$, $y = 0.194$, $z = 0.194$
4 O_{II} in 4(*e*): $x = 0.403$, $y = 0.694$, $z = 0.306$.

The occurrence of metal atom doublets in the MoO_2 structure type is of considerable interest and the findings of Zachariasen [4] that there exist dioxides of technetium and rhenium belonging to this type adds to the importance of this structural feature. However, the latter compounds could only be prepared in powder form, and the powder photographs were too complicated to allow a determination of the atomic positions to be made [5].

Recently, one of the present authors (G.A.) in the course of studies on the vanadium-oxygen system has performed a complete structure determination for vanadium dioxide [6]. The unit cell dimensions are similar to those of the dioxides of MoO_2-type (*cf.* Table 1) and the atomic arrangement is in close agreement with the idealized structure of molybdenum dioxide given above.

VO_2: Space-group: $P2_1/c$

4 V in 4(*e*): $x = 0.242$, $y = 0.975$, $z = 0.025$
4 O_I in 4(*e*): $x = 0.10$, $y = 0.21$, $z = 0.20$
4 O_{II} in 4(*e*): $x = 0.39$, $y = 0.69$, $z = 0.29$.

No reflexions, indicating a deviation from the symmetry $P2_1/c$, could be observed for vanadium dioxide and it is obvious that this compound may be considered as representing the idealized form of the "MoO_2 structure type".

The occurrence of metal atom doublets in molybdenum and wolfram dioxide has been discussed by Pauling [9], who suggests that each of the quadrivalent metal atoms has a tendency to use its two remaining valence electrons for the formation of a double bond with another metal atom. The influence of the number of valence electrons on the bond length within the doublets is illustrated in Table 2. The data given for technetium and rhenium dioxide are calculated assuming the metal atom positions of these compounds to be equal to those of molybdenum dioxide, which assumption seems to be justified by the close similarity of the lattice dimensions. The longer metal-metal atom distances within the strings of MeO_6 octahedra are very nearly the same for the various dioxides of the MoO_2 structure type but considerably longer than the corresponding distance in the titanium dioxide (rutile) structure. A detailed comparison, however, is rendered a little difficult by the fact that the metal

Table 1. Unit cell dimensions of dioxides of the MoO_2 structure type and comparable data for titanium dioxide (rutile). Space-group $P2_1/c$ *(No. 14).*

Compound	*a* Å	*b* Å	*c* Å	*β*	Reference
TiO_2(rutile)	5.918	4.593	5.464	122°79	2
VO_2	5.743	4.517	5.375	122°61	7
MoO_2	5.584	4.842	5.608	120°94	8
WO_2	5.565	4.892	5.650	120°69	8
TcO_2	~5.53	~4.79	~5.53	~120°	4
ReO_2	5.562	4.838	5.561	120°87	5

Table 2. Some interatomic distances of dioxides of the MoO_2 structure type and of titanium dioxide (rutile).

Compound	Number of valence electrons of metal	Me-Me distances within strings of MeO_6 octahedra	O-O distance along edge common to two MeO_6 octahedra of Me atom doublet	Reference
TiO_2 (rutile)	4 + 0	2.959	2.520	2, 3
VO_2	4 + 1	2.65; 3.12	2.62	6
MoO_2	4 + 2	2.50; 3.10	3.1	1, 8
WO_2	4 + 2	2.49; 3.08		1, 8
TcO_2	4 + 3	(2.48); (3.06)		4
ReO_2	4 + 3	(2.49); (3.08)		5

atoms considered belong to different periods of the periodic system. With decreasing bond distance within the metal atom doublets, the distance between the oxygen atoms of the edge common to the two MeO_6 octahedra is markedly increased.

It might be possible to obtain further knowledge of the bond conditions of compounds of this type by studying mixed oxides, *e. g.* of the system titanium dioxide-vanadium dioxide. Such experiments will be started in the near future. A detailed account of the determination of the structure of vanadium dioxide will shortly appear elsewhere.

These studies form part of a research program on metal oxides and related compounds financially supported by the *Swedish Natural Science Research Council.*

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Studies on Molybdenum and Molybdenum Wolfram Oxides of the Homologous Series Me_nO_{3n-1}

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Members of the homologous molybdenum and molybdenum wolfram oxide series Me_nO_{3n-1} with n equal to 8, 9, 10, 11, 12 and 14 have been prepared. The phase relations of the series have been studied as a function of the wolfram to molybdenum ratio and of the temperature. The former has been found to determine the value of n in the first place, higher homologues forming with increasing relative contents of wolfram.

A comparison is given between previously derived unit cell dimensions for the homologues and those actually observed for preparations of various compositions.

Studies of some chemical properties have been found to indicate that the structural uniformity of the series also corresponds to a very similar chemical behaviour.

The existence of a series of structurally interrelated molybdenum and molybdenum wolfram oxides of the general formula Me_nO_{3n-1} (Me = Mo, W) was recently reported by one of the present authors [1]. Previously known members of this series were the molybdenum oxides Mo_8O_{23} and Mo_9O_{26} [2]. It was found that several molybdenum wolfram oxides of about the same metal to oxygen ratio of 1 : 2.9 are built analogously and detailed structure determinations were carried out for the two double oxides $Me_{10}O_{29}$ and $Me_{11}O_{32}$ [3]. The investigation was facilitated by a general discussion of the geometrical properties of structures of this type [4].

A brief description of the structure of *e. g.* $Me_{10}O_{29}$ will serve to illustrate the building principles of this oxide series. (*Cf.* Fig. 1.) The structure may be considered as built up of blocks of ReO_3-type, *i. e.* three-dimensional aggregates of metal-oxygen octahedra joined by sharing corners in a basically cubic arrangement. The blocks are of infinite extension in two dimensions and have a characteristic width of ten octahedra in a third direction. The actual MeO_6 octahedra are distorted, the metal atoms forming puckered networks and the oxygen polyhedra being modified to a square pyramidal arrangement with a remote oxygen atom completing the octahedra. The blocks are mutually

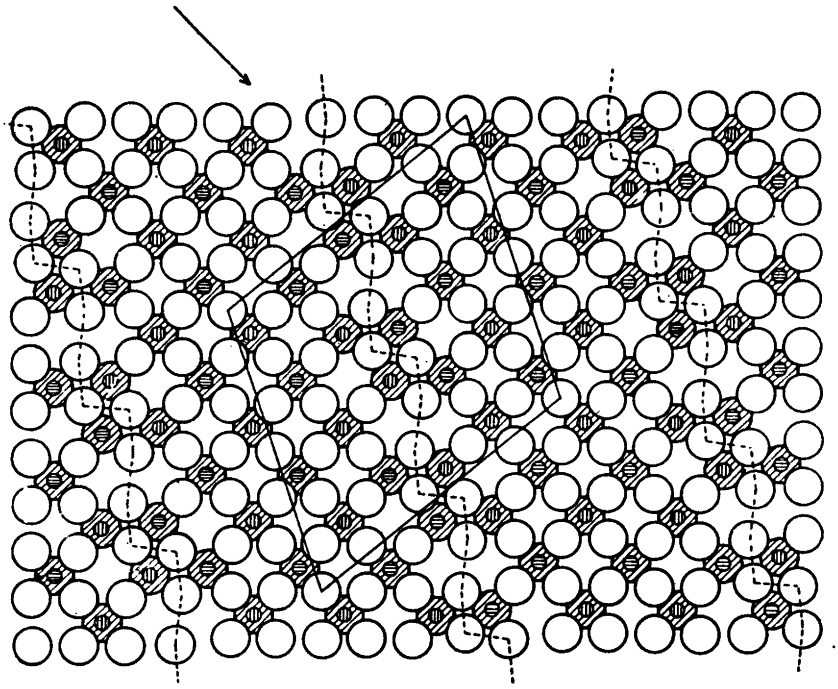

Fig. 1. Structure of the phase $Me_{10}O_{29}$ of the homologous series Me_nO_{3n-1}. The metal atoms (small circles) are slightly displaced normally to the figure, the direction of the displacement being indicated by the different shadowing. The arrow shows the direction of the finite extension of the ReO_3 type blocks, which are indicated by dashed lines.

connected along characteristically folded planes by MeO_6 octahedra sharing edges. The various members of the series, Me_nO_{3n-1}, differ concerning the number of MeO_6 octahedra (n) determining the finite extension of the blocks. It was suggested that this series of compounds, expressible by a general formula and built on common structural principles, be characterized as a *homologous series.*

Further studies on this series have now revealed the existence of the two oxides $Me_{12}O_{35}$ and $Me_{14}O_{41}$ and given some knowledge of the formation conditions and stability of the various phases. Exact unit cell dimensions have been determined and compared with the ideal ones.

EXPERIMENTAL METHODS

The samples were generally prepared by heating weighed mixtures of molybdenum metal, molybdenum trioxide and wolfram trioxide in evacuated, sealed silica tubes for a couple of days. The starting materials were molybdenum powder (Kahlbaum) which had been heated *in vacuo* at about 400° C, molybdenum trioxide (Baker) dried at 400° C and wolframic acid (Kahlbaum) which had been heated for several hours at about 850° C.

Due to the considerable volatility of molybdenum trioxide at the heating temperatures it was necessary to keep the temperature as constant as possible and to minimize the temperature gradient in the silica tubes. The tubes were thus kept in narrow holes, bored in a heavy cylinder of fire-resisting steel, which was covered by a steel lid. The cylinder was heated in a vertical electric furnace. A platinum-platinum rhodium thermocouple was introduced in one of the holes of the cylinder through a narrow opening in the lid. The cold junction of the thermocouple was kept at 0° C in a Dewar flask containing ice and water. A constant voltage, just insufficient to give the temperature wanted was applied to the furnace while a small additional voltage was controlled by the thermocouple using a Wheelco regulator. In this way the variations of temperature were reduced to about ± 2° at a heating temperature of 600—950° C. The samples were quenched in water from the reaction temperature.

In spite of these precautions it was often found difficult to obtain reproducible results when preparing and heat treating the pure molybdenum oxides. It was found that the phase and stability relations in the molybdenum oxygen system are even more complicated than has been known previously. Further studies on this subject are in progress and a note on some preliminary results has recently appeared elsewhere [5]. It was found that equilibrium was much more quickly attained in samples containing wolfram. For high wolfram contents, however, the reaction rate was rather low and high temperatures had to be used in order to reach equilibrium within a reasonable period of time.

The samples were investigated by taking X-ray powder photographs in a Guinier focusing camera of 80 mm diameter using monochromatized Cu-$K\alpha$ or Cr-$K\alpha$ radiation. The very low background of these photographs greatly facilitated the observation of extraneous phases. The powder patterns were evaluated according to the method given by Hägg [6], which introduces an automatic correction for film shrinkage. In order to increase the accuracy, sodium chloride (a = 5.6398 Å) was always added to the powder specimens as an internal standard. Single crystals from various preparations were also investigated by taking rotation and Weissenberg photographs with Cu-K radiation.

PHASE ANALYSIS

Mixtures of the gross composition $Mo_{1-x}W_xO_{2.9}$ were heated at temperatures between 650° and 950° C for a couple of days. The preparations obtained in this way consisted of dark blue or blue-violet crystals. With low contents of wolfram, the crystals often appeared as aggregates of needles or prisms, grown together parallel to the needle axis. The needle-shape of these species, however, was frequently destroyed due to a very marked cleavage at right angles to the needle axis. The cleavage surface showed a very high reflexion power. With higher contents of wolfram, the crystals were smaller and the needle-shape was more pronounced.

The X-ray powder patterns of the substances investigated were rather complicated throughout. The preliminary analyses of the samples thus had to be carried out by means of photographs of selected single crystals. These diagrams could generally be interpreted by comparison with the ideal structural data previously derived for the various members of the Me_nO_{3n-1} series [4]. The approximate unit cell dimensions thus obtained made possible the interpretation of the powder photographs. Samples containing molybdenum wolfram oxides of this homologous series were found to be fairly homogeneous since, in general, only a few, very weak lines of extraneous phases were observed in the powder patterns.

The results of the phase analysis are illustrated in Fig. 2, which gives a tentative diagram of the phase relations of the homologous series $(Mo_{1-x}, W_x)_n O_{3n-1}$ as a function of the value of x and of the formation temperature.

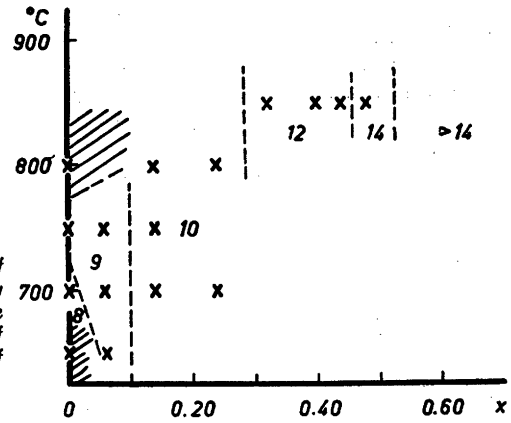

Fig. 2. Tentative diagram showing phases of the homologous series Me_nO_{3n-1}, obtained by heating samples $Mo_{1-x}W_xO_{2.9}$. Crosses denote specimens investigated and figures values of n. *Shadowed areas indicate occurrence of phases not belonging to this series.*

While the phases Mo_8O_{23} and Mo_9O_{26} — often contaminated by other oxides — could be prepared by heating samples not containing wolfram at rather restricted intervals of temperature around 700° and 750° C respectively, the presence of a small proportion of wolfram ($x = 0.06$) was found to bring about the formation of pure Me_9O_{26} phase within the approximate temperature region 650°—750° C. The phase $Me_{10}O_{29}$ was obtained by heating samples with x equal to 0.14 and 0.24 at temperatures between 700° and 800° C while preparations with x values of 0.32—0.44 and 0.48 heated at 850° C were identified as consisting of the phases $Me_{12}O_{35}$ and $Me_{14}O_{41}$ respectively. With higher contents of wolfram, still higher members of the homologous series were obtained. However, the identity of the phases could no longer be determined with certainty, due to the continually increasing similarity between the powder patterns of the higher homologues.

Attempts to prepare the phases $Me_{11}O_{32}$ and $Me_{13}O_{38}$ in a similar way at 750° C and higher temperatures were not successful. The former compound,

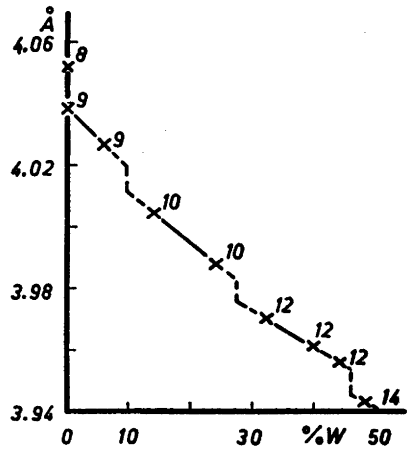

Fig. 3. Length of b *axis of phases of the homologous series $(Mo, W)_nO_{3n-1}$ as a function of the wolfram content.*

however, was obtained as a small amount of tiny, thin crystal needles together with a major constituent consisting of a micro-crystalline substance of the approximate composition $MoWO_6$ when heating an equimolecular mixture of molybdenum trioxide and wolfram trioxide in an evacuated, sealed silica tube at about 700° C for 45 days [3,7]. As this experiment was carried out without special precautions to ensure a constant temperature within the reaction tube, the actual molybdenum to wolfram ratio of the crystal needles is unknown.

The experiments obviously show that the relative wolfram content is a fundamental factor determining the width of the ReO_3-type blocks of the oxide formed. The influence of the formation temperature is obvious for the pure molybdenum oxides but seems to be of minor importance for the mixed oxides within the range of temperature investigated. This might be more fully elucidated by extensive studies of samples prepared at lower temperatures. Due to the low reaction rate even at moderate wolfram contents, such an investigation would be rather tedious. However, the observations reported above might indicate that phases Me_nO_{3n-1} with n are even more stable at high temperatures than those with n odd.

UNIT CELL DIMENSIONS

The fundamental role played by the ReO_3 type building unit in the structures of this oxide series manifests itself as a pronounced substructure effect in the X-ray diffraction patterns. Since the metal atoms of the ReO_3 type blocks form puckered layers (*cf.* Fig. 1), the subcell corresponds to two units of MeO_3 and shows (psuedo)tetragonal symmetry with $a' \approx c' \approx d\sqrt{2}$ and b' (tetragonal axis) slightly longer than d (d = the space diagonal of a regular MeO_6 octahedron) [4]. All strong reflexions of the various members of the homologous series appear in multiplets close to the reciprocal lattice points of the subcell.

The substructure effect causes the X-ray patterns of the homologues to be very similar, the similarity of the diagrams increasing with increasing n. In spite of their considerable complexity, it was possible to interpret the powder photographs of the phases with $n = 8$, 9, 10, 12, and 14 up to fairly high diffraction angles and to derive unit cell dimensions of preparations of various compositions. Representative parts of the powder patterns are listed in Table 1.

Table 2 gives a comparison between the unit cell dimensions thus obtained and the ideal ones, calculated on the basis of the values of $d = 3.750$ Å for the length of the space diagonal of a regular MeO_6 octahedron and of $\delta = 0.21$ for the parameter introduced to allow for the mutual repulsion of the metal atoms of octahedra joined by sharing edges. (The formulae used when calculating the ideal dimensions are given in reference [4].) The accuracy of the observed cell data may be estimated to be better than $\pm$ 0.01 Å for the a and c axes, $\pm$ 0.002 Å for the b axis and $\pm$ 0.02° for the monoclinic angle. The data given in this paper for Mo_8O_{23} and Mo_9O_{26} are thus somewhat more exact than those previously reported [8].

The agreement between observed and ideal values for the length of the a axes is good, the difference amounting to 0.11 Å (0.65 %) for Mo_8O_{23} and

Table 1. Part of the powder diffraction patterns of homologues of the series Me_nO_{3n-1}. Cu-Kα radiation.
(Reflexions marked with an asterisk coincide with other lines of the powder patterns.)

Tetragonal subcell		Me_8O_{23} (0 % W)				Me_9O_{26} (0 % W)				$Me_{10}O_{29}$ (14 % W)				$Me_{12}O_{35}$ (32 % W)				$Me_{14}O_{41}$ (48 % W)			
$h'k'l'$	$\sin^2\Theta$ calc	I	hkl	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc	I	hkl	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc	I	hkl	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc	I	hkl	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc	I	hkl	$\sin^2\Theta$ obs	$\sin^2\Theta$ calc
$10\bar{1}$	.0423	v st	$20\bar{3}$	.0510	.0510	v st	$20\bar{4}$	.0498	.0499	v st*	$20\bar{5}$	.0490	.0490	st	$20\bar{5}$	.0474	.0475	v st*	$20\bar{5}$	.0463	.0466
		st	$20\bar{2}$	.0298	.0298	m+	$20\bar{3}$	.0310	.0311	st—	$20\bar{4}$	.0321	.0321	m*	$20\bar{4}$	.0335	.0335				
		m	$20\bar{1}$	.0158	.0158	m—	$20\bar{2}$	.0178	.0179	w+	$20\bar{3}$	.0194	.0197	w	$20\bar{3}$	.0225	.0226				
		m	401	.0333	.0334	w	400	.0339	.0340	m*	$40\bar{1}$	.0343	.0344	w	401	.0350	,0351				
		st	402	.0378	.0379	st	401	.0386	.0387	st	400	.0391	.0391	st	402	.0394	.0395	st	404	.0400	.0399
101	.0423	st*	403	.0495	.0495	st	402	.0490	.0490	v st	401	.0483	.0484	st	403	.0470	.0470	v st*	405	.0463	.0463
						w	$60\bar{3}$	.0937	.0935	v w	$60\bar{5}$	.0921	.0924								
200	.0845	st	600	.0815	.0815	st	$60\bar{2}$	.0821	.0822	st	$60\bar{4}$	.0825	.0825	v st	$60\bar{2}$	.0825	.0825	st*	600	.0831	.0829
		w	204	.0538	.0539	v w	204	.0573	.0574	w	204	.0599	.0600								
002	.0845	st	205	.0830	.0830	m+	205	.0837	.0837	st	205	.0838	.0839	st	207	.0834	.0835	st*	209	.0831	.0834
		m +*	206	.1194	.1194	v w	206	.1157	.1156	w	206	.1122	.1123								
		w	$40\bar{6}$	.2038	.2038	w	$40\bar{8}$	.1995	.1998	v w*	$40\bar{1}0$	.1960	.1961					v w	$40\bar{1}0$	.1863	.1862
$20\bar{2}$	.1690	v st*	$40\bar{5}$	.1580	.1579	st+	$40\bar{7}$	.1591	.1593	v st	$40\bar{9}$	.1599	.1600	st	$40\bar{9}$	.1606	.1606	m*	$40\bar{9}$	.1613	.1612
202	.1690	v st	805	.1712	.1711	st+	803	.1725	.1725	v st	801	.1725	.1727	v st*	805	.1718	.1715	st	809	.1715	.1714
010	<.0423	v st*	010	.0361	.0362	v st*	010	.0364	.0364	v st	010	.0371	.0371	v st	010	.0376	.0376	v st	010	.0382	.0382
011	<.0634	st*	112	.0495	.0497	w	112	.0508	.0508	w+	112	.0521	.0521	w	113	.0533	.0535	v w	114	.0546	.0547
		m*	113	.0660	.0661	w	113	.0653	.0653	m—	113	.0651	.0651	w+ *	114	.0643	.0639	w	115	.0635	.0639
		w	$31\bar{1}$	.0649	.0649	w	$31\bar{2}$	.0640	.0641	w	$31\bar{3}$	.0638	.0638								
110	<.0634	w	310	.0566	.0566	w	$31\bar{1}$	.0569	.0570	w+ *	$31\bar{2}$	.0577	.0577	m	311	.0582	.0583	m	310	.0588	.0589
		m	311	.0554	.0554	m—	310	.0555	.0555	w+	$31\bar{1}$	.0561	.0561					v w	311	.0574	.0573

Table 2. Observed and ideal unit cell dimensions of members of the series Me_nO_{3n-1}.

Phase	% W	*a* Å		*b* Å		*c* Å		*β*		*c* sin *β* Å		
		obs	ideal	obs	ideal	obs	ideal	obs	ideal	obs	ideal	Δ (ideal-obs)
Me_8O_{23}	0	16.88	16.77	4.052	>3.750	13.39	13.82	73°81	73°88	12.86	13.27	0.41
Me_9O_{26}	0	16.80	16.77	4.039	>3.750	14.58	15.00	95°43	94°56	14.51	14.95	0.44
»	6	16.82	16.77	4.027	>3.750	14.59	15.00	95°47	94°56	14.53	14.95	0.42
$Me_{10}O_{29}$	14	16.78	16.77	4.005	>3.750	17.43	17.76	111°74	110°51	16.19	16.63	0.44
»	24	16.83	16.77	3.988	>3.750	17.45	17.76	111°73	110°51	16.21	16.63	0.42
$Me_{11}O_{32}$	32	16.80	16.77	3.970	>3.750	19.61	20.00	89°25	88°61	19.61	19.99	0.38
	40	16.81	16.77	3.961	>3.750	19.62	20.00	89°26	88°61	19.62	19.99	0.37
»	44	16.80	16.77	3.956	>3.750	19.64	20.00	89°23	88°61	19.64	19.99	0.35
$Me_{14}O_{41}$	48	16.78	16.77	3.943	>3.750	24.04	24.43	73°19	72°87	23.01	23.35	0.34

being less (0.01—0.07 Å) for the other preparations. The extension of the lattice in this direction is very little influenced by the value of n and the relative content of wolfram.

The c axes and monoclinic angles observed are in fair agreement with the ideal values. A spectacular idea of their combined deviation is obtained by comparing the observed and ideal values of the product $c\sin\beta$, representing the finite width of the ReO_3-type blocks. The observed widths are throughout less than the ideal ones. These differences cannot be removed by altering the value of the parameter δ in the formula for the ideal unit cell dimensions (*i. e.* by assuming other interatomic distances between the metal atoms of the MeO_6 octahedra joined by edges) as this would give rise to considerable discrepancies between the observed and ideal metal atom coordinates [4]. The deviations between the observed and ideal widths of the ReO_3-type blocks must evidently be due to a contraction in this direction as compared with the extension in the direction of the a axis. It is noteworthy that the absolute value of the contraction is fairly constant irrespective of the value of n and thus of the width of the blocks.

The variation of the length of the b axis is of special interest as the increase of this dimension in comparison with the space diagonal of the regular MeO_6 octahedron is associated with the puckering of the metal atom layers and the distortion of the oxygen polyhedra. The values of b (*cf.* Fig. 3) decrease with increasing wolfram content. The introduction of wolfram atoms in the structures evidently causes a modification of the metal-oxygen polyhedra towards a more regular octahedral arrangement. It is of interest in this connection to notice that the structure of the wolfram oxide $W_{20}O_{58}$ belonging to the series Me_nO_{3n-2} is not puckered and is very probably built up of fairly ideal WO_6 octahedra [9]. The length of the b axis of this compound amounts to 3.767 Å [8]. The variation of the b axis dimension of the Me_nO_{3n-1} oxides is discontinuous when passing from one homologue to another.

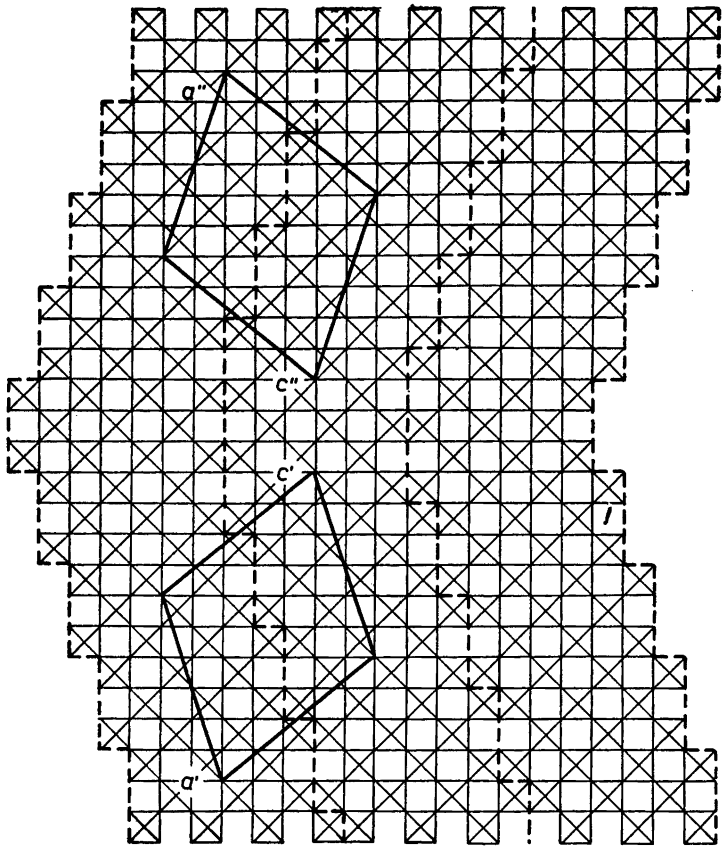

Fig. 4. Twinning of $Me_{10}O_{29}$ (idealized diagram).

TWINNING

As mentioned above the crystals often appear in aggregates, the crystal individuals having grown together parallel to the monoclinic axis. Weissenberg photographs of a crystal of the phase $Me_{10}O_{29}$ revealed the existence of twinning, the angle between the a axes of the twins amounting to 144°. This indicates the twinning to be caused by a change of the slope of the ReO_3-type blocks in the way illustrated in Fig. 4, $(30\bar{2})$ being the twin plane. The ideal value according to this scheme for the angle between the a directions of the twin individuals is 143.°12 ($= 2\,\beta''$ in the notation used in the discussion of the geometry of these substances [4]). This angle will have the same value also for twins of other members of the homologous series. The general indices for the twin plane of the homologue Me_nO_{3n-1} ($n = 3p+q$, p and q integers)

will be (100) for $q = -1$, $(30\bar{1})$ for $q = 0$, and $(30\bar{2})$ for $q = 1$ or generally $(30\ \overline{q+1})$.

Growing together of crystal individuals not governed by a twin law has also been observed.

CHEMICAL PROPERTIES

According to Glemser and Lutz[10], Mo_8O_{23} ("β-molybdenum oxide") and Mo_9O_{26} ("β'-molybdenum oxide") differ considerably in their chemical properties, the former being soluble in dilute ammonia while the latter is not affected by this reagent. Treating a mixture of the two oxides with ammonia was suggested by these authors as a means of preparing Mo_9O_{26} in a pure state.

In view of the close structural relationship a considerable difference between the chemical properties of the two oxides seemed rather improbable. Pure samples (a few crystals) of these compounds and also of the higher homologues were thus tested with various reagents, *viz.* hydrochloric acid, sulphuric acid, nitric acid, sodium hydroxide, and ammonia. The samples were observed under the microscope. In no case could an obvious attack be noticed even after a few hours. A preparation containing ζ-molybdenum oxide[5] was, however, dissolved by ammonia. It thus seems probable that the "β-molybdenum oxide" of Glemser and Lutz, is identical with the ζ-oxide while their β'-oxide is Mo_8O_{23}, Mo_9O_{26} or a mixture of these two phases.

The authors wish to thank Professor G. Hägg for his kind interest in this investigation. The work forms part of a research program on metal oxides and related compounds financially supported by the *Swedish Natural Science Research Council.*

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The Magnetic Properties of the Red and Green Nickel-Compounds of the Type $[Ni^{(PE_3)_2}_{X_2}]$. Studies in Magnetochemistry 13 *

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The red complex compound $[Ni(PE_3)_2Cl_2]$ is diamagnetic having $\chi_M = -202 \times 10^{-6}$. The transplanar molecule is in a singulet state (dsp^2-bonds). The green compound $[Ni(PE_3)_2(NO_3)_2]$ follows the Curie-law $\chi_M^{corr} = \frac{1.194}{T}$ from 85°K to 400°K. Extrapolation to 0°K gives the Weiss correction $\Delta = 0$. The permanent magnetic moment is 3.10 Bohr magnetons. This molecule is in a triplet state (sp^3-bonds or ion-dipole bonds).

Red complex nickel-compounds of the type $[Ni(PR_3)_2 X_2]$ were prepared and described by K. A. Jensen [1]. X is Cl, Br, or I and R is C_2H_5, C_3H_7, or C_4H_9. The dipole moments of the compounds $[Ni(PPr_3)_2 Cl_2]$ and $[Ni(PE_3)_2 Br_2]$ are according to Jensen practically 0 indicating transplanar configuration of the molecules. No *cis*-forms could be isolated. However, a green nitrate $[Ni(PE_3)_2 (NO_3)_2]$ could be prepared having the dipole moment 8.85 *D*. A corresponding red nitrate does not seem to exist. The magnetic measurements carried out by Asmussen [2] on Jensen's preparates show that the compounds $[Ni(PE_3)_2 Cl_2]$ and $[Ni(PPr_3)_2 Cl_2]$ are diamagnetic having the gram susceptibilities $-0.32_8 \times 10^{-6}$ (19° C) and $-0.34_2 \times 10^{-6}$ (20° C), respectively. The measurements of the dielectric and the magnetic susceptibilities show that we have an undoubted conformity between experiment and the magnetic criterion for planar configuration. Furthermore the magnetic measurements show that the green nitrate is paramagnetic. The effective moment was determinded to 3.05 Bohr magnetons at 19° C. According to the magnetic criterion we may expect this molecule to have tetrahedral configuration (sp^3-bonds or ion-dipole-bonds). As the dipole moment measurement cannot distinguish between *cis*-planar and tetrahedral configuration we may assume

* No. 12 of this series was published in the *Proceedings of the Symposium on Coordination Chemistry*, Copenhagen 1954 (p. 27).

that these measurements and the magnetic data support each other as far as the tetrahedral configuration is concerned.

K. A. Jensen [1] observed that the solution containing nickel nitrate and alkyl-phosphine is temporarily coloured red before the green nitrate separates. K. A. Jensen suggests that this colour change originates in the transformation of the red but instable *trans*-form into the stable green nitrate. We have observed that after having been held a few degrees above the melting point and then suddenly cooled down to liquid air temperature the green nitrate contains a few red particles. Furthermore we observed that solutions of the green nitrate in methyl- or ethylalcohol are decomposed slowly but the colour changes to red when the solutions are cooled in carbondioxide-acetone. If a few crystals of the green compound are placed in water they are rapidly dissolved and decomposed but in a zone near the crystal surface a red colour is observed even at room temperature. Solutions in benzene are rather stable and their green colour is preserved at low temperature. In view of these observations we considered it of interest to prepare the compounds again and study their magnetic properties, in particular the temperature dependence of the susceptibility in order to decide whether a transformation between the two magnetically different forms occurs. The temperature dependence of the susceptibilities has not been studied before.

We prepared the red [Ni $(PE_3)_2Cl_2$] and the green [Ni $(PE_3)_2(NO_3)_2$] following the methods of K. A. Jensen [1]. The compounds were recrystallized and analysed. Nickel was determined cyanometrically. The melting points were 112—113° and 130—131° C, respectively. The results of the analysis together with room temperature measurements of the susceptibilities of these compounds at different values of the field strength are given in Table 1. The magnetic measurements were carried out as reported in earlier papers [3].

Table 1. Field strength dependence of the susceptibility. Analysis.

$\left[\mathrm{Ni}\ \begin{matrix}(PE_3)_2\\Cl_2\end{matrix}\right]$, red. 297°K			$\left[\mathrm{Ni}\ \begin{matrix}(PE_3)_2\\(NO_3)_2\end{matrix}\right]$, green. 296°K		
H (Ørsteds)	$-\chi_g \times 10^6$	$-\chi_M \times 10^6$	H (Ørsteds)	$\chi_g \times 10^6$	$\chi_M \times 10^6$
6 226	0.549	201	2 657	9.195	3 853
7 021	0.554	203	3 346	9.146	3 832
7 635	0.556	203	3 822	9.177	3 845
Mean value	0.553	202	Mean value	9.172	3 843
Analysis Ni %	Calc. 16.04 % Found 16.14 %, 16.08 %		Analysis Ni %	Calc. 14.01 % Found 14.07 %	

The susceptibilities for the two salts are independent of the field strength; their analytical and magnetical purity is secured. Table 2 gives the measurement of the temperature dependence of the susceptibility of the red compound from liquid air temperature to room temperature.

Table 2. $\left[\mathrm{Ni} \begin{matrix} (\mathrm{PE_3})_2 \\ \mathrm{Cl_2} \end{matrix} \right]$. *Temperature dependence of* χ.

°K	$-\chi_g \times 10^6$	$-\chi_M \times 10^6$
297	0.553	$202._0$
195	0.546	$199._8$
85	0.539	$197._3$
Mean value	0.546	$199._7$

The susceptibility is independent of the temperature. The slight decrease in χ_g when the temperature increases is not significant. The mean value -0.55×10^{-6} differs rather much from the earlier value. However, discre-

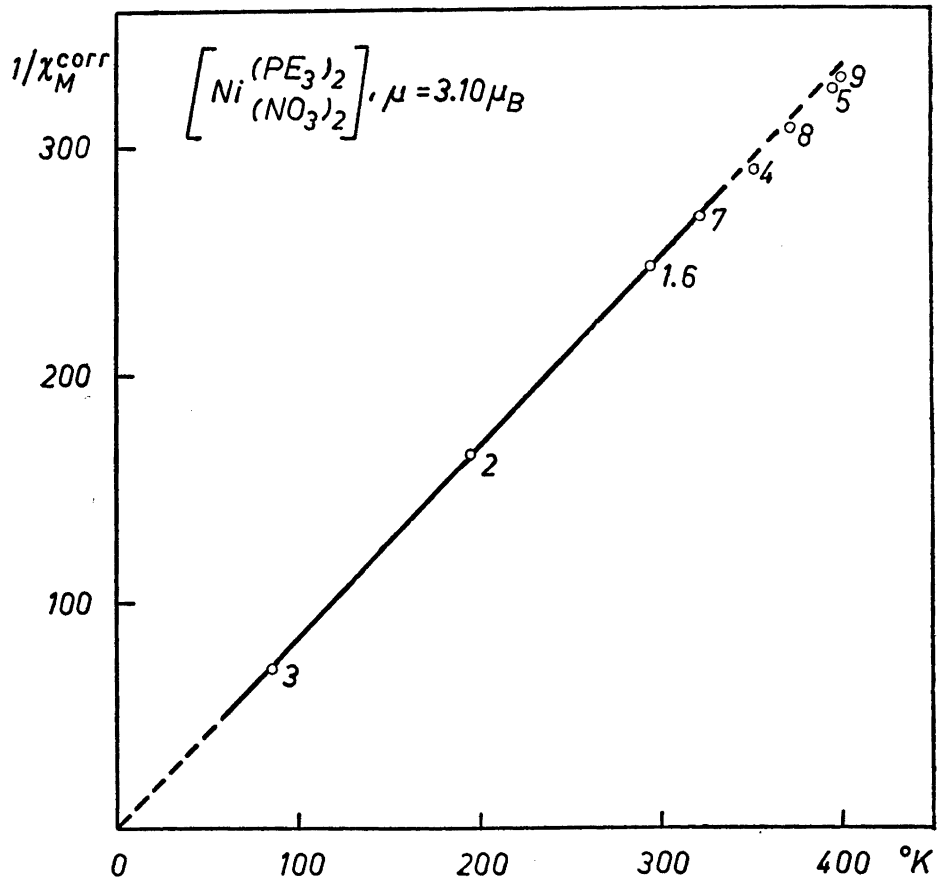

Fig. 1. $\left[Ni \begin{matrix} (PE_3)_2 \\ (NO_3)_2 \end{matrix} \right]$. *Reciprocal corrected molar susceptibility plotted against the abs. temperature (°K).*

pancies of this order of size in the case of diamagnetic compounds are not unusual for different specimens of the same compound (different origin). We did not consider the effect important enough to recrystallize the salt until the diamagnetic susceptibility was constant. The original sample was not available; it might have contained a paramagnetic nickelcomplex. The new value, -0.55×10^{-6}, is considered to be the most probable.

The temperature-susceptibility measurements for the green nitrate are given in Fig. 1 where the reciprocal corrected molar susceptibilities are plotted against the temperature. We applied the molar diamagnetic susceptibility (room temperature) of the red chloride to correct the molar susceptibility of the green nitrate for diamagnetism. The total molar diamagnetic correction is $-\chi \times 10^6 = 202 - 42 + 38 = 198$, using the values -21×10^{-6} and -19×10^{-6} for the gramionic susceptibilities for the ions Cl^- and NO_3^-, respectively (compare Asmussen [2]).

The green nitrate follows the Curie-law exactly; the Weiss correction is 0. The equation for the straight line is

$$\chi_M^{corr} = \frac{1.194}{T}$$

The magnetic moment calculated by means of the Curie-constant measured is = 3.10 Bohr magnetons. The earlier determination of the effective moment is in good agreement with this value; the deviation is not greater than that to be expected for specimens of different origin. The points in Fig. 1 are numbered, the measurements were carried out in the succession indicated by these numbers. It will be seen that points above 325°K are situated a little below the Curie-line. The reason is that the green nitrate gives off a slight amount of phosphine, which is condensed as small droplets in the measuring tube; the tube was evacuated at room temperature and sealed off. The series of measurements show, however, that the reaction is reversible.

The general conclusions of the measurements here reported are: The *trans*-planar molecule is in a singulet state and the tetrahedral molecule is in a triplet state. No significant indications of magnetically observable reactions in the solid state have been found. The colour changes in solution may be attributed to chemical reactions in the liquid.

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Short Communications

Studies on the Chemistry of Lichens

IX.* On the Identity of Ocellatic acid and Thamnolic acid

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The lichen *Pertusaria corallina* (Ach) was investigated by Hesse [1] who isolated from it a phenolic acid of low solubility called ocellatic acid, m. p. 208° (decomp.) which analysed for $C_{21}H_{18}O_{12}$ and contained a methoxyl group. An acid, evidently identical with ocellatic acid has now been isolated from *P. corallina* which grows abundantly in the vicinity of Stockholm. The substance was conveniently crystallised from dioxan and after drying analysed for $C_{19}H_{16}O_{11}$. This formula agrees with that of thamnolic acid, m. p. 223° (decomp.), isolated by Zopf from *Thamnolia vermicularis* (Sw.) Ach. and investigated in detail by Asahina *et al.* who found it to be a depside of structure I (for references see[2]). The only known derivative of thamnolic acid melting without decomposition is the dimethyl ester (m. p. 158°) obtained in small yields by Asahina *et al.* through cautious methylation with diazomethane. When methylated according to Asahina, ocellatic acid gave a dimethyl ester of m. p. 157—158°.

For direct comparison thamnolic acid was isolated from *Thamnolia vermicularis*. It crystallised with one molecule of dioxan, melted at 210—212° (decomp.) and gave the same colour reactions as ocellatic acid. The two substances also proved identical on paper chromatography [3]. A full proof of their identity was obtained by comparing their infra-red spectra in the region 5—10 μ where both acids gave the same characteristic bands.

The identity of ocellatic and thamnolic acid gives further proof to the wide distribution in lichens of the latter, formerly believed to occur only in *Thamnolia* and in members of the family *Cladoniae*. The occurrence of thamnolic acid in another *Pertusaria* species, *P. dealbeata* Nyl., was recorded by Koller and Hamburg [4].

Experimental *. *Ocellatic acid.* *P. corallina* (160 g) was extracted with ether in a Soxhlet-apparatus for several days yielding 4.5 g of a crude acid which proved to be homogeneous on paper chromatography [3]. $R_F = 0.06$ on paper impregnated with 0.1 M Na_2HPO_4 (*n*-butanol-water) and $R_F = 0.15$ on unimpregnated paper (*n*-butanol-ethanol-water 4:1:5). The chromatograms were observed in ultra-violet light (greenish white spots) and sprayed with a solution of *p*-phenylenediamine (yellow spots) or *bis*-diazotised benzidine (red spots).

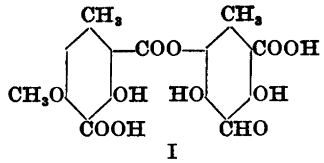
For analysis the acid was crystallised several times from dioxan yielding prisms of a faintly yellowish colour containing dioxan of crystallisation, completely lost only after drying *in vacuo* at 100°; m. p. 221—223° (decomp.). Loss of weight, found: 16.8 %. $C_{19}H_{16}O_{11}$, $C_4H_8O_2$, requires 17.3 % dioxan. Found: C 54.3; H 3.91; OCH_3 7.36. $C_{19}H_{16}O_{11}$ requires C 54.3; H 3.82; OCH_3 7.37.

The dimethyl ester was prepared according to Asahina *et al*[5]. by adding the calculated amount of ethereal diazomethane to a supersaturated solution of the acid (170 mg) in acetone (500 ml) at —15°. Prisms (30 mg) from benzene-ethanol, m. p. 157—158°. Found: OCH_3, 20.4. $C_{21}H_{20}O_{11}$ requires $(OCH_3)_3$ 20.8.

Thamnolic acid. *Thamnolia vermicularis (sensu* Asahina) which is known to contain only thamnolic acid was picked out by its dark blue colour under the ultra-violet light from a mixture with *Thamnolia subvermicularis*

* Part VIII. *Acta Chem. Scand.* **9** (1955) 917.

* All melting points uncorrected.



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(Asahina) which contains squamatic and baeomycessic acids and which shows a quite distinct bright white fluorescence. By extracting the pure lichen (10 g) with ether in a Soxhlet-apparatus, crude thamnolic acid (1 g) was obtained. It was chromatographically indistinguishable from ocellatic acid and was obtained from dioxan as prisms containing one mole of dioxan, lost after drying *in vacuo* at 100°. Found: Loss of weight: 17.0 %. $C_{19}H_{16}O_{11},C_4H_8O_2$ requires 17.3 % dioxan. Found: Equiv.wt. 205. $C_{19}H_{16}O_{11}$ requires equiv.wt. 210.1.

The author is indebted to Docent R. Santesson, Botaniska Museet, Upsala, for collecting the *Thamnolia* mixture and to Fil.lic. K. E. Almin, Svenska Träforskningsinstitutet, Stockholm, for performing the infra-red spectra.

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The Standard Oxidation Potential of the System, 1,4-Naphthoquinone/H+ — 1,4-Naphthohydroquinone and the Solubility of 1,4-Naphthohydroquinone, 1,4-Naphthoquinone and 1,4-Naphthoquinhydrone

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Previously LaMer and Baker have determined by electrometric titration the standard oxidation potential of the system 1,4-naphthohydroquinone = 1,4-naphthoquinone + $2H^+$ + 2e at 25° C. They found the value 0.4698 ± 0.0002 volt [1]. Many other values are reported in the literature, but they have all been determined in non-aqueous solutions, and are therefore incomparable to the value here reported.

The solubility of 1,4-naphthohydroquinone, 1,4-naphthoquinone and 1,4-naphthoquinhydrone has not been determined before. The solubilities of 1,4-naphthoquinone and 1,4-naphthohydroquinone presented in this paper have been determined directly, that of 1,4-naphthoquinhydrone has been calculated as shown below.

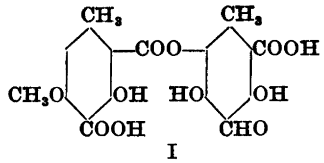
The standard oxidation potential presented in this paper for the system in question at 20° C in an aqueous solution of 0.01 M HCl + 0.09 M KCl was determined in two ways (see Table 1) combined with the determination of the solubility of 1,4-naphthoquinone and of 1,4-naphthohydroquinone.

Denoting the electromotive forces of the three cells by E_A, E_B and E_C, respectively we have the following equations: (the liquid-liquid junction potential being taken as zero)

$\pi_0' - E_A = \pi_0$, $\pi_0' - E_B = \pi_B$ and $\pi_0' - E_C = \pi_C$, where π_0' and π_0 are the standard oxidation potentials of the systems benzoquinone—benzohydroquinone and 1,4-naphthoquinone — 1,4-naphthohydroquinone, respectively; π_B and π_C are the oxidation potentials at pH = 0 in the left half-cells of (B) and (C). π_0 and the solubility product of 1,4-naphthoquinhydrone L may be calculated from π_B, π_C and the solubilities of 1,4-naphthoquinone (c_{oQu}) and 1,4-naphthohydroquinone (c_{oHy}):

$$\pi_B = \pi_0 + 0.02905 \log c_{oQu}/c_{Hy} \text{ and}$$
$$\pi_C = \pi_0 + 0.02905 \log c_{Qu}/c_{oHy} \text{ and}$$
$$c_{Qu}c_{oHy} = c_{oQu}c_{Hy} = L$$

c_{Hy} and c_{Qu} are the concentrations of 1,4-naphthohydroquinone and 1,4-naphthoquinone in the left half-cells of (B) and (C), respectively. The following results were obtained: (π_0' is taken as 0.7028 volt [2]) $c_{oQu} = (1.099 \pm 0.005)\ 10^{-3}$ mole/liter, $c_{oHy} = (5.25 \pm 0.03)\ 10^{-3}$ mole/liter. $E_A = 0.2211 \pm 0.0005$ volt, $E_B = 0.2092 \pm 0.0001$ volt, $E_C = 0.2735 \pm 0.0001$ volt. The value of π_0 calculated by means of π_0' and E_A is 0.4817 ± 0.0005 volt. The value of π_0 calculated by means of π_0', E_B, E_C, c_{oQu}, and c_{oHy} is 0.4812 ± 0.0002 volt. The calculated value for L and the solubility of 1,4-naphthoquinhydrone are



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The standard oxidation potential presented in this paper for the system in question at 20° C in an aqueous solution of 0.01 M HCl + 0.09 M KCl was determined in two ways (see Table 1) combined with the determination of the solubility of 1,4-naphthoquinone and of 1,4-naphthohydroquinone.

Denoting the electromotive forces of the three cells by E_A, E_B and E_C, respectively we have the following equations: (the liquid-liquid junction potential being taken as zero)

$\pi_0' - E_A = \pi_0$, $\pi_0' - E_B = \pi_B$ and $\pi_0' - E_C = \pi_C$, where π_0' and π_0 are the standard oxidation potentials of the systems benzoquinone—benzohydroquinone and 1,4-naphthoquinone — 1,4-naphthohydroquinone, respectively; π_B and π_C are the oxidation potentials at pH = 0 in the left half-cells of (B) and (C). π_0 and the solubility product of 1,4-naphthoquinhydrone L may be calculated from π_B, π_C and the solubilities of 1,4-naphthoquinone (c_{oQu}) and 1,4-naphthohydroquinone (c_{oHy}):

$$\pi_B = \pi_0 + 0.02905 \log c_{oQu}/c_{Hy} \text{ and}$$
$$\pi_C = \pi_0 + 0.02905 \log c_{Qu}/c_{oHy} \text{ and}$$
$$c_{Qu}c_{oHy} = c_{oQu}c_{Hy} = L$$

c_{Hy} and c_{Qu} are the concentrations of 1,4-naphthohydroquinone and 1,4-naphthoquinone in the left half-cells of (B) and (C), respectively. The following results were obtained: (π_0' is taken as 0.7028 volt [2]) $c_{oQu} = (1.099 \pm 0.005)\ 10^{-3}$ mole/liter, $c_{oHy} = (5.25 \pm 0.03)\ 10^{-3}$ mole/liter. $E_A = 0.2211 \pm 0.0005$ volt, $E_B = 0.2092 \pm 0.0001$ volt, $E_C = 0.2735 \pm 0.0001$ volt. The value of π_0 calculated by means of π_0' and E_A is 0.4817 ± 0.0005 volt. The value of π_0 calculated by means of π_0', E_B, E_C, c_{oQu}, and c_{oHy} is 0.4812 ± 0.0002 volt. The calculated value for L and the solubility of 1,4-naphthoquinhydrone are

Table 1. The standard oxidation potential was determined in two ways:

1) by measuring the electromotive force of the cell (A)

Pt	1,4-Naphthoquinhydrone (cryst.) 0.01 M HCl + 0.09 M KCl	0.01 M HCl 0.09 M KCl	Benzoquinhydrone (cryst.) 0.01 M HCl + 0.09 M KCl	Pt	(A)

2) by measuring the electromotive force of the cells (B) and (C)

Pt	1,4-Naphthoquinone (cryst.) 1,4-Naphthoquinhydrone (cryst.) 0.01 M HCl + 0.09 M KCl	0.01 M HCl 0.09 M KCl	Benzoquinhydrone (cryst.) 0.01 M HCl + 0.09 M KCl	Pt	(B)

and

Pt	1,4-Naphthoquinhydrone (cryst.) 1,4-Naphthohydroquinone (cryst.) 0.01 M HCl + 0.09 M KCl	0.01 M HCl 0.09 M KCl	Benzoquinhydrone (cryst.) 0.01 M HCl + 0.09 M KCl	Pt	(C)

$(4.51 \pm 0.07)\ 10^{-7}$ (mole/liter) [2] and $(6.72 \pm 0.05)\ 10^{-4}$ mole/liter, respectively.

Experimental. Chemicals (all meltings points uncorrected):

1,4-Naphthoquinone was purified by distillation with steam and recrystallisation from ethanol; m. p. 125°—126° C.

1,4-Naphthohydroquinone was prepared by reduction of 1,4-naphthoquinone with stannous chloride and recrystallisation from water. The product was preserved in a sealed glass tube filled with nitrogen; m. p. 191°—192° C.

1,4-Naphthoquinhydrone was prepared by mixing concentrated solutions of 1,4-naphthoquinone and 1,4-naphthohydroquinone in ethanol. The product was recrystallised from ethanol, and preserved in a sealed glass tube filled with nitrogen.

Benzoquinhydrone was recrystallised from ethanol.

The solubility measurements.

The solubility of 1,4-naphthoquinone. The aqueous solution of 0.01 M HCl + 0.09 M KCl was transferred into two 250 ml flasks each of which was closed with a rubber stopper mounted with a Jena 33 G 4 gas filter dipping in the solution, and a glass tube with a stopcock. In order to free the solution from oxygen, carbon dioxide was led through for about 30 min. The carbon dioxide was washed in a titanium trichloride solution containing a citrate buffer. After the removal of oxygen 0.5 g of 1,4-naphthoquinone was suspended in each flask.

In order to reach equilibrium both from the side of supersaturation and from the side of subsaturation one of the flasks was kept at 40° C for one hour before being placed in the thermostat, where they were rotated at 20.00 ± 0.02 ° C. With aid of carbon dioxide under pressure samples were obtained by forcing the solution through the filter into a 15 ml automatic Knudsen-pipette. As burette was used a Krogh-syringe mounted with a micrometer screw. The content of quinone was titrated with titanium trichloride and indigodisulfonate as indicator.

Samples were analysed after the lapse of 4 hours, 25 hours, 48 hours and 72 hours. The process, supersaturation — saturation, proceeds very slowly. It took between 48 and 72 hours before equilibrium was reached. On the other hand the equilibrium of the process, subsaturation — saturation, is reached in the course of 4 hours.

The solubility of 1,4-naphthohydroquinone. The same procedure as that described above was used, except that equilibrium was established only from the side of subsaturation. The content of hydroquinone was titrated with ammonium ferricsulfate and potassium rhodanide as indicator.

The electrometric measurements. The apparatus are earlier described [3]. A Wolff compensator with a mirror galvanometer was used. The position of the mirror was read by telescope. As standard cells were used two Weston cells compared to each other. The sensibility was 0.1 mV.

Care was taken to avoid oxygen in the cells. The electrodes were washed with the aqueous solution of 0.01 M HCl + 0.09 M KCl. This was freed from oxygen by bubbling-through nitrogen which previously had been passed

over pieces of copper wire heated to some 500° C.

The electromotive forces of the cells and the time passed since the last washing of the electrodes were measured. Cell (B) and (C) became constant in 20 hours since the last washing of the left electrodes had been effected.

The work has been completed at Laboratoriet for Fotokemi, Fotografi og Reproduktionsteknik, Technical University, Copenhagen. The authors thanks are due to dr. E. Güntelberg for information and help in the work.

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Paper Chromatography of Nucleoside Polyphosphates

ROLF BERGKVIST and ADAM DEUTSCH

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Paper chromatography according to the method of Hanes and Isherwood [1], namely descending chromatography with *n*-propanol-ammonia (*d* 0.880)-water (60:30:10) separates the 5′-mono-, di- and triphosphates of any one of the ribonucleosides adenosine, guanosine, inosine, cytidine and uridine from each other. On the other hand, difficulties are generally encountered on attempts to separate by this method mixtures which contain phosphates of the different nucleosides. Some time ago, the need arose in this laboratory for the resolution of mixtures containing different adenosine and inosine 5′-phosphates and a method for the separation of such mixtures by two-dimensional paper chromatography was devised [2]. In the first direction, the chromatogram was developed with *n*-propanol-ammonia-water, separating the individual members in each series and in the second direction with a solvent system consisting of saturated ammonium sulphate solution-water-*iso*-propanol (79:19:2), which had been introduced [3] for the separation of the products of hydrolysis of nucleic acids. It was found that this solvent separates the two nucleoside series of phosphates from each other.

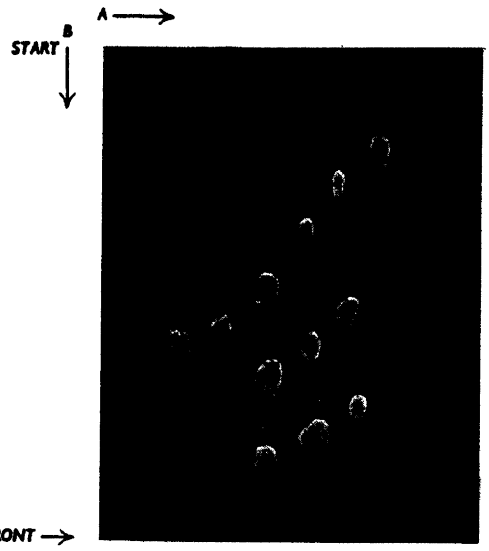

Fig. 1. Two-dimensional chromatogram (Whatman No. 1 paper) of a mixture of AMP, ADP, ATP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP and UTP, approximately 50 µg of each compound. (A) first solvent: n-*propanol-ammonia-water (60:30:10), 60 hours. (B) second solvent: saturated ammonium sulphate solution-water-*iso-*propanol (79:19:2), 10 hours. (1) AMP; (2) ADP; (3) ATP; (4) GMP; (5) GDP; (6) GTP; (7) IMP; (8) IDP; (9) ITP; (10) UMP; (11) UDP; (12) UTP.*

In the course of recent work on the isolation of the 5′-triphosphates of guanosine [4,5] and uridine [4,6] from muscle, the ammonium sulphate-water-*iso*propanol solvent system proved to be useful for the separation of the 5′-triphosphates of different nucleosides including those of guanosine, uridine, adenosine, inosine and xanthosine. We have therefore applied the previously described method of two-dimensional paper chromatography to the resolution of mixtures containing the 5′-mono-, di- and triphosphates of adenosine (AMP, ADP and ATP), guanosine (GMP, GDP and GTP), inosine (IMP, IDP and ITP) and uridine (UMP, UDP and UTP) and obtained adequate resolution of all the twelve components (Fig. 1). On the other hand,

over pieces of copper wire heated to some 500° C.

The electromotive forces of the cells and the time passed since the last washing of the electrodes were measured. Cell (B) and (C) became constant in 20 hours since the last washing of the left electrodes had been effected.

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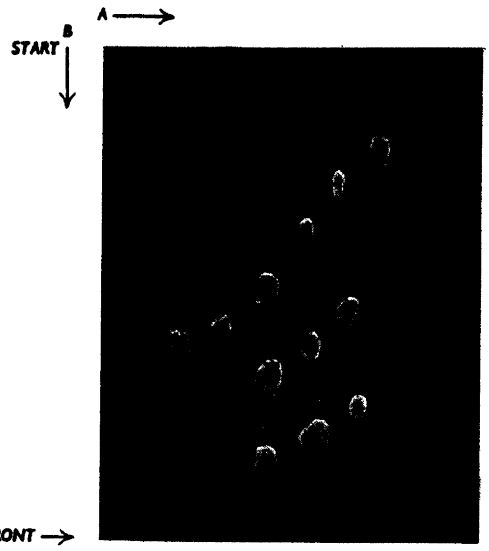

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the 5′-mono-, di- and triphosphates of cytidine (CMP, CDP and CTP) are not separated by this method from the corresponding 5′-phosphates of uridine.

Experimental. Materials. The adenosine and inosine polyphosphates [7] and the cytidine, guanosine and uridine polyphosphates [5,6] were prepared and purified as described previously, except for CMP and UMP which were obtained by courtesy of Prof. A. R. Todd, Cambridge. *Method* as described in previous communications for qualitative [2] purposes and for analysis [8] on the eluted spots.

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Isotope Effect in the Hydrolysis of Grignard Compounds

LARS OLOF ASSARSSON

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Hydrolysis of Grignard compounds of the type RMgX by water containing heavy hydrogen isotopes has been carried out, mostly for preparative purposes, by several investigators. In these reactions the water has usually been present in a deficient or approximately theoretical quantity.

In the present investigation Grignard compounds have been hydrolyzed by a large excess of tritiated water. Under such conditions, one might be able in a simple way to calculate the ratio k_T/k_H between the reaction rates of tritium and protium, respectively, and further, to draw conclusions about the reaction mechanism. Water has been present in an approximately tenfold excess over the Grignard compound, and therefore the reaction in practice must be expected to take place according to

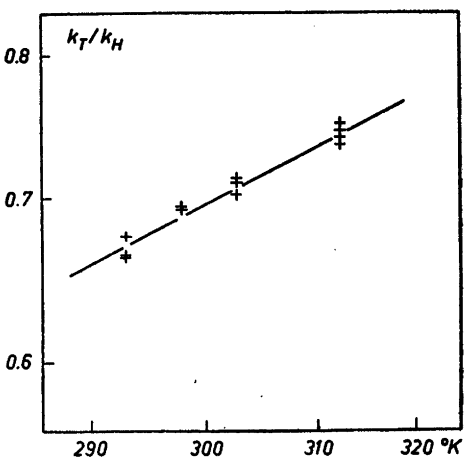

Fig. 1. The ratio k_T/k_H (logarithmic scale) for methane formation as a function of the absolute temperature (inverted scale.)

$$RMgX + H_2O \rightarrow RH + Mg\begin{matrix}X\\OH\end{matrix}$$

especially as no precipitates of basic magnesium salts are formed in the solvents used. Such precipitates include water and in this way withdraw it from the reaction with RMgX, and if the amount of water present is deficient both hydrogen atoms will react [1]. The reaction may also be assumed to take place practically completely with regard to RMgX.

In the present experiments on the hydrolysis of methylmagnesium and phenylmagnesium iodide, the Grignard compound, dissolved in anisole, was added to a solution of water in pyridine by means of an injection syringe or by a dropping funnel. The anisole as well as the pyridine had been dried according to current methods and afterwards tested for moisture by the usual Zerewitinoff method. In the case of C_6H_5MgJ a fraction of the solvent was

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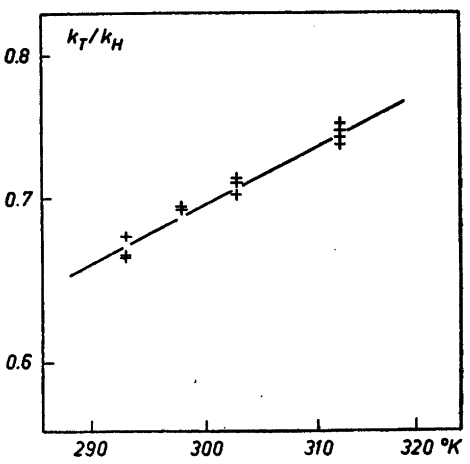

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The hydrocarbons obtained in the decompositions were burned over cupric oxide, and the radioactivity of the water obtained as well as that of the initial water used in the reaction was determined by means of a Geiger-Müller counter [2].

Some experiments with methylmagnesium iodide were carried out in order to check the influence on the isotope effect of the concentration of the Grignard reagent, the rate of addition, the diameter of the syringe summit, and the conditions of drying the methane. Provided the addition rate was low enough and the summit sufficiently thin, reproducible results were obtained, and the concentration of the Grignard reagent was found to be of little importance.

With methylmagnesium iodide the syringe addition method was used, and decompositions were carried out at different temperatures, 20°, 25°, 30° and 40° C, for the measurement of the temperature coefficient of k_T/k_H and in order to give an estimation of the difference between the apparent activation energies of the tritium and protium reactions. The results are given in Fig. 1, where k_T/k_H is plotted on a logarithmic scale as a function of the (inverted) absolute temperature. The slope of the line corresponds to a difference in the apparent energy of activation of about 1 kcal/mole.

At 20° C, where k_T/k_H for the methane formation is 0.67 ± 0.01 that for the benzene formation was found to be 0.61 ± 0.03. In the latter experiments the addition was carried out drop by drop. This is probably one reason why the experimental irregularities are larger, and it is not certain, indeed, whether there is a real difference between the two figures given.

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Experiments on the same reactions, carried out in other, more indifferent solvents are made, the intention being also to extend the investigations to the formation of other hydrocarbons than methane and benzene.

Added in proof: A review[3] of the scarce experimental facts concerning this type of reaction has been published after the accomplishment of this paper.

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Calculation of the Directive Influence of a Nitro Group in Position 2 in Thiophene

LARS MELANDER

Nobel Institute of Chemistry, Stockholm 50, Sweden

In many respects thiophene is very similar to benzene, the sulphur atom being a substitute for a $-CH=CH-$ fragment of the latter. According to Longuet-Higgins [1] the π-electron structure of thiophene might be treated in substantially the same way as that of benzene, the sulphur atom providing two orbitals of pd^2 type instead of the two missing carbon p-orbitals. The only difference from benzene in the MO LCAO treatment, then, is that the resonance and overlap integrals between adjacent orbitals belonging to atoms of different kinds are assumed to be smaller than those between orbitals of atoms of the same kind, the latter integrals being assumed to have the same values as in benzene. The magnitudes of the former integrals, supposed to be proportional to one another, were found to be 80 % of the latter from the requirement that the resonance energies of thiophene and benzene, experimentally 30 and 40 kcal $mole^{-1}$, respectively, should be in the experimental ratio. The value 20 kcal $mole^{-1}$ for thiophene, recently proposed by Sunner [2], will reduce the figure to about 60 %.

The picture of the π-electron system of thiophene referred to makes it an intermediate between those of benzene and butadiene plus ethylene, and consequently an entering substituent will prefer the position close to sulphur, irrespective of whether the attack is electrophilic, radical,

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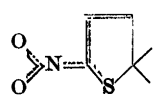
Table 1. (For explanations, see the text.)

Compound	Position	Kind of attack	Polarization energy		
			$x=1$ [5]	$x=0.8$	$x=0.6$
Thiophene [4]	2	el., rad., nucl.	1.849	1.678	1.523
2-Nitrothiophene	5	electrophilic	1.861	1.694	1.544
		radical	1.809	1.636	1.480
		nucleophilic	1.757	1.578	1.416
Thiophene [4]	3	el., rad., nucl.	1.849	1.851	1.837
2-Nitrothiophene	4	el., rad., nucl.	1.852	1.856	1.845

or nucleophilic [3,4]. When there is already a *meta*-directing substituent, such as a nitro group, in the 2-position, however, the question arises as to the point of the next attack. The sulphur atom tends to direct it towards the 5-position, but by analogy with benzene the nitro group would be expected to tend to direct it towards the 4-position.

In connexion with some experimental work in progress, it was found of interest to apply the method of Wheland [5] to the thiophene model of Longuet-Higgins and calculate the "polarization energies", *i. e.* the π-electron energy of the two hypothetical substitution intermediates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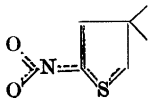
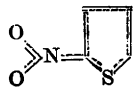


relative to the initial molecule



(In these figures the extension of the π-electron system is indicated by dotted lines.) As other magnitudes involved could be assumed to be mainly independent of the position, the intermediate of lowest energy could be expected to correspond also to the lowest activation energy and hence to the most rapid reaction. It should be emphasized, however, that the intermediate is not identical with the activated complex of the reaction, the latter being passed before the former is reached, at least in ordinary nitrations [6], but their energy heights will generally be correlated.

The calculations were carried out in exactly the same way as those of Wheland [5], and with the same values of the quantum mechanical parameters of the nitro group. The results are given in Table 1, where x denotes the assumed ratio between the two kinds of exchange and overlap integrals mentioned above. The column for $x = 1$ will consequently correspond to a perfect benzene ring, and these values are found in Wheland's paper. For comparison, the values previously computed for unsubstituted thiophene [4] have also been included. The energy unit is Wheland's $-\beta_0$, a positive quantity equivalent to about 38 kcal mole $^{-1}$.

In a very recent paper [7], which appeared after the present calculations were completed, Matlow and Wheland give a somewhat modified set of parameters for the nitro group. These minor changes are not believed to influence the general conclusions which could be drawn from Table 1.

The results are just what could be expected from a superposition of the two directing influences, the much stronger directing power of the sulphur atom even at x as high as 0.8 being predominant. The agreement with existing experimental facts [8], which cannot be expected to be better than qualitative, seems to be satisfactory.

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Note on the Crystal Structure of Niobium Dioxide *

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Niobium dioxide was prepared by heating an intimate mixture of niobium metal powder and niobium pentoxide in a sealed, evacuated silica tube at about 1 250° C for eight days and also by reducing niobium pentoxide with hydrogen at about 1 120° C. X-ray powder photographs were taken in a Guinier focusing camera of 80 mm diameter using Cu$K\alpha$ radiation. Potassium chloride (a = 6.293 Å) [1] was added to the specimens as an internal standard. The diagrams given by the two samples were found to be identical and in fair agreement with the data reported by Brauer [2].

The results confirmed the statement of Brauer that all reflexions of major intensity are consistent with a rutile type lattice and that, in addition, a considerable number of weak reflexions is present. In order to account for these superstructure lines it was first attempted to interpret the powder pattern on the basis of a monoclinic unit cell of MoO_2-type [3]. This turned out to be impossible and it was thus obvious that the kind of distortion of the rutile type structure present in niobium dioxide is different from that found in the dioxides of several transition elements of the fifth, sixth and seventh groups of the periodic system, *viz.* vanadium [4], molybdenum [5], wolfram [5], technetium [6] and rhenium [6].

Table 1 gives part of the powder photograph of niobium dioxide. It is compatible with a tetragonal unit cell with the dimensions $a = 2\sqrt{2}a_r = 13.71$ Å and $c = 2c_r = 5.985$ Å, $a_r = 4.846$ Å, and $c_r = 2.993$ Å being the dimensions of the subcell of rutile type. This suggests a unit cell content of 32 formula units of NbO_2 (calculated density 5.90, observed density 5.98). It must be very difficult to find the structural details of this compound from powder data. Attempts to prepare samples suitable for investigations by single crystal methods have so far been unsuccessful.

Table 1. Part of powder photograph of niobium dioxide. CuKα radiation. (The reflexions marked with (r) are compatible with the basic rutile type structure.)

I	$\sin^2\Theta_{obs}$	hkl	$\sin^2\Theta_{calc}$
vw	0.0197	101	0.0198
vw	0.0323	211	0.0324
w	0.0451	301	0.0451
st+	0.0507	400 (r)	0.0506
w	0.0577	321	0.0577
vw	0.0702	411	0.0703
st	0.0918	222 (r) 520	0.0917
w	0.0956	501 431	0.0956
m	0.1011	440 (r)	0.1012
vw	0.1083	521	0.1083
w	0.1169	402 (r)	0.1170
vw	0.1264	620 (r)	0.1265
vvw	0.1462	541	0.1462
vw	0.1522	103	0.1525
vw	0.1588	631	0.1589
vw	0.1649	640 213	0.1644 0.1651
vw	0.1712	701	0.1715
vw	0.1774	303	0.1778
w	0.1839	612 730 721	0.1833 0.1834 0.1842
vw	0.1903	323	0.1904
st	0.1929	622 (r) 650	0.1928 0.1929
m	0.2025	800 (r)	0.2024

These studies form part of a research program on metal oxides and related compounds financially supported by the *Swedish Natural Science Research Council.*

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Two Protein Fractions of Barley, Precipitated from Acid Solutions

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When water extracts of barley or malt (pH 5.8—8.0, +20° C, 0.1 % sodium bromate present) are acidified to pH 4.5—4.0, a brownish protein precipitate is obtained, designated in the following as fraction I. A white protein, called fraction II, is precipitated from the supernatant by addition of sodium chloride to a concentration of 2 moles per liter. When prepared from cold, clear-filtered extracts, these precipitates are almost completely redissolvable at pH 7.

Both these fractions are precipitated by ammonium sulphate in the saturation range 15—40 % (at pH 6.0), but the precipitates obtained in this way are largely insoluble. Thus, it is possible that a part of the proteins in these fractions has escaped the attention of numerous investigators of barley proteins. It may be mentioned, that Quensel [1] discarded the globulin fraction precipitated by 15—40 % saturated ammonium sulphate (at pH 7.0), as he found this fraction very heterogeneous in the ultracentrifuge.

I. *Fraction I* is a protein complex containing associated material of acid character primarily, *viz.* nucleic acids, polyphenols and lipids. The amount of lipids and especially that of nucleic acids in fraction I is greatly increased when the extraction is performed at pH 7—8 instead of 5.8. Calcium ion in the concentration of 0.01 M inhibits the extraction of nucleoproteins and partially that of lipoproteins.

The nitrogen content of fraction I varies from 12.5 to 14 % and phosphorus content from zero to 0.6 %. The amount of lipids (determined as ether soluble substance from extracts with alcohol-ether, dried in vacuo) varies from about 3 % in preparations from extracts by 0.01 M calcium sulphate to nearly 20 % from extracts by distilled water adjusted to pH 8.0.

Attempts to estimate the distribution of phosphorus in fraction I according to Schneider [2] has given the following results: 1) The lipids, extracted with an alcohol-ether mixture, contain only traces of phosphorus. 2) About 20—25 % of the phosphorus is soluble in cold 5 % trichloroacetic acid (TCA) and thus may derive from phytic acid and inositol esters [3]. 3) The phosphorus remaining after treatment with cold TCA is released nearly quantitatively by the same reagent at +90° C. Thus 70—80 % of phosphorus in fraction I is probably due to nucleic acids [2,4]. The presence of nucleic acids is also verified by precipitating the protein moiety by sodium dodecyl sulphate [5] and measuring the light adsorption of the supernatant between 220 and 300 mμ. 4) Only traces of phosphorus remain in the precipitate after treatment with heated TCA-solution. The amount of phosphoproteins may thus be regarded as negligible.

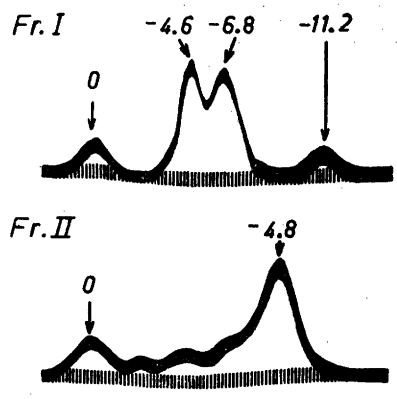

Fig. 1. Electrophoretic patterns of fractions I and II. Phosphate buffer, $\mu = 0.1$, pH = 7.7. Potential gradients 5.3 volt cm^{-1} (fraction I) respectively 6.9 volt cm^{-1} (fraction II). Exposures after 120 minutes. The numbers indicate ionic mobilities, 10^{-5} cm^2 volt^{-1} sec^{-1} as unit.

The electrophoretic pattern of fraction I is shown in Fig. 1. Despite the great complexity of the fraction the electrophoretic pattern is rather simple and depends very little on the pH-value at the extraction.

Paper electrophoretic experiments have shown that the component with the mobility −6.8 units contains the coloured complexes and lipids. The rapd component with mobility −11.2 units is stained with bromophenol blue like proteins and is found even in phosphorus free preparations.

II. *Fraction II*, precipitated as described above, contains as an impurity some material ressembling fraction I. It can be purified by suspending the centrifuged pre-

cipitate with about a 20-fold volume of water, adjusting the pH value first to 7.0 and then to 4.5, centrifuging off the grayish precipitate and reprecipitating the white protein with 2.0 M sodium chloride. Maximum precipitation seems to occur between pH 3.5 and 4.0. Potassium chloride is equally effective as sodium chloride, but ammonium sulphate at the same ionic strength (0.67 M, 16 % saturation) precipitates fraction II only incompletely. Potassium iodide, which has a solubilizing effect on cereal proteins, does not precipitate any protein from this fraction.

Fraction II contains 15.5—16 % N, when prepared from barley and 14.5—15 % N, when prepared from malt. It contains 1.3 % of sulphur, no phosphorus and no or only traces of lipids (at the highest 2 %).

The electrophoretic pattern of fraction II is shown in Fig. 1. The amount of the principal component varies in different preparations from 75 to 90 %. This component seems to be heterogeneous, especially when prepared from barley.

When fraction I is washed or dissolved and reprecipitated, a white precipitate ressembling (also electrophoretically) fraction II is obtained from the supernatant by 2.0 M sodium chloride. Thus it may be suggested that fraction I partially consists of complexes, whose protein moiety is similar to fraction II.

Both fractions I and II are resistant to high temperatures and remain soluble in 1 % solutions at +100° C for more than two hours at pH 6.5 (μ = 0.01), but at pH 6.0 fraction II begins to coagulate slowly after 15 minutes. Similar fractions (if not identical) can be precipitated from heated extracts (*e. g.* from brewery worts).

The amount of these fractions in barley and malt extracts varies depending on the extraction conditions. As much as 31 % of the total nitrogen of barley extract at pH 8.0 is found in fraction I and about 16 % in (crude) fraction II. In extracts obtained at pH 5.8 the corresponding values are 16 and 14 %, respectively, in calcium-containing extracts at pH 7.0, 12 and 11 %. From malt extracts these fractions are obtained in amounts of the same order of magnitude as from barley extracts, but owing to the greater amount of total nitrogen the relative values are smaller.

The classification of these proteins according to the scheme of Osborne is difficult. Perhaps they might be regarded as globulins, because they are partially precipitated by dialysis. Fraction II, however, is slowly rendered insoluble by dialysis. As heat resistant proteins they are neither albumins, nor can they be glutelin or hordein fractions, because their amide nitrogen content is only 9—10 % of their total nitrogen (determined according to Mecham and Olcott [6]).

The experience in the preparation of these fractions has shown that such common standard methods as precipitation by ammonium sulphate and dialysis are not always ideal. It can be argued that the rather low pH-values used may denature proteins but judging from the solubility of the obtained precipitates the denaturation, if any, is not so great as after ammonium sulphate precipitation.

Further examination is needed to decide, if these fractions contain some earlier characterized barley proteins, *e. g.* some of the globulins defined by Quensel [1]. At present it is not possible to state, whether the nucleoprotein and lipoprotein complexes present in fraction I are naturally occurring in barley grain or if they are artefacts resulting from the extraction.

The author wishes to thank Dr S. Brohult, Mr. H. Nihlén and Dr E. Sandegren for valuable advices and discussions.

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On the Molecular Weight of Potassium Hyaluronate from the Vitreous Body of Cattle

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It has been reported by Blix and Snellman [1], Meyer and Palmer [2], and very recently by Laurent [3] that hyaluronate from the vitreous humor has a much lower viscosity than hyaluronate from human umbilical cord, and consequently a lower molecular weight.

Using streaming birefringence measurements, Blix and Snellman [1] and Brunish and Rowen [4] estimated a molecular weight of vitreous hyaluronate at less than 250×10^3, and about 500×10^3, respectively, while Laurent [3] found $340-500 \times 10^3$ by the light scattering technique.

In this institute we have determined the molecular weight of a preparation of vitreous hyaluronate by means of the osmometrical technique described by the present authors [5].

The cattle eyes were enucleated just after killing, divided into two parts by an equatorial cut, cleaned from surrounding, especially retinal tissues, minced with a homogenizing tube, and filtered through eight layers of gauze. Potassium hyalnuroate was isolated according to Jensen [6].

The nitrogen contents determined after Kjeldahl, and the sulphur contents determined after Bürger's [7] method modified by Zimmermann [8] were 3.10 % and less than 0.1 %, respectively. The relative viscosity of the preparation dissolved in McIlvaine buffer (pH = 7) was measured at 3.8 in a Dalgaard-Mikkelsen-Kvorning microviscometer. The result of the osmometrical measurement is in Fig. 1. For details of this technique confer Christiansen and Jensen [5].

The value obtained, 270×10^3, seems small compared with some of the figures cited above, *e. g.* those obtained by Laurent; however, we may consider our statement to be realistic on account of the supposed polydispersity of hyaluronate from the vitreous body. The molecular weights obtained with the aid of light scattering are weight averages like those obtained from ultracentrifugation, while

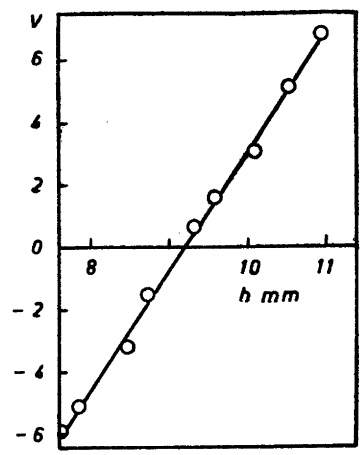

Fig. 1. The graph from which the molecular weight is calculated. The inner liquid contained 10.12 mg in 1 ml of the buffer solution which was used as the outer liquid.

those obtained from osmometrical measurements are number averages. Consequently the light scattering values tend to be high relatively to the osmometrical values if the solution contains a small fraction of molecules much larger then the average.

Laurent himself points out that the value obtained by him is probably too high because of the polydispersity of the employed solution, judged from the scattering envelope. Earlier investigators (Blix and Snellman [1], Brunish and Rowen[4]) have also reported solutions of *corpus vitreum* hyaluronate to be polydisperse.

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The Electrical Conductivity of the System CaO-CaF_2

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In studies of the system $CaO-CaF_2$[1], the electrical conductivities of the different mixtures were measured. The results are shown in Fig. 1 for the temperatures 1 500, 1 515, 1 530 and 1 545 ° C, all well over the melting points. The interesting thing is that in spite of its well-known low conductivity [2] ($\varkappa$ = 0.175 ohm^{-1} cm^{-1} at the melting point 2 570 ° C) CaO in these mixtures increases the conductivity. We also see that at a certain point on an isotherm, the conductivity rapidly increases and at a still higher percentage of CaO suddenly decreases.

The reasons for these very interesting features can be explained if we remember that the ionic radii of F^- and O^{2-} are nearly equal (r_{F^-} = 1,36 Å and $r_{O^{2-}}$ = 1.40 Å [3]). At lower percentages of CaO we can suppose that the oxygen ions can be built in into the "liquid lattice" of the fluorine ions, similarly to what happens in the solid state in mixed crystals of KCl-$SrCl_2$[4], where the cation vacancies produced by the unequal charges of the cations increase the ionic conduction. In our case the unequal charges of O^{2-} and F^- will also cause anion vacancies and an increase in the conduction. A consequence of these vacancies must be a lowering of the density of the melt. This lowering is a linear function of the composition. If we know the density of molten CaF_2, we can compute what densities the melts should be without vacancies and with vacancies. In Table 1 this has been done for 1 545 ° C

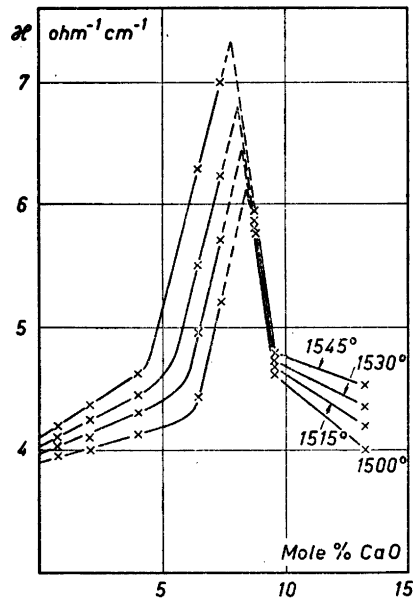

Fig. 1. Electrical conductivities at different temperatures.

Table 1.

Mole fraction CaO	Densities observed	Densities computed without vacancies	Densities computed with vacancies
0.0000	2.75	2.75	2.75
0.0205	2.68	2.73	2.68
0.0400	2.59	2.72	2.62
0.0650	2.53	2.70	2.53
0.0735	2.50	2.69	2.51
0.0956	2.63	2.68	2.44

and compared with observed values. We see that the agreement is excellent up to x_{CaO} = 0.0735. But at x_{CaO} = 0.0956 the observed density disagrees and is much higher than the computed. If we return to Fig. 1 it is seen than there is an increase in the conductivity with CaO concentration — caused by the movement of fluorine ions through the vacancies — at the beginning, there is a slight linear rise which is however followed by a more rapid increase at higher concentrations. The concentration of these vacancies has a limit, after which this type of "liquid lattice" breaks down and the conductivity decreases and the density increases. This maximum number of vacancies decreases with increasing temperature, *i. e.* with greater thermal agitation.

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Metal Ammine Formation in Solution

IX. Heats and Entropies of Successive Steps in the Formation of Nickel(II) and Copper(II) Ethylenediamine and Trimethylenediamine Complexes

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Estimation of heats of reaction from temperature coefficients in systems with several consecutive constants demands a high precision of the measurements, and with many authors this fact has led to inconsistent results due to an overestimate of the data. In this paper we have, therefore, determined the heats by calorimetric titration of solutions in which the distribution of the complexes are known from affinity measurements. The nickel(II) and copper(II) systems with ethylenediamine (en) and trimethylenediamine (tn) were examined. The heats of successive steps were found to be rather constant. This means that the variation in free energy with the number of ligands taken up is mainly determined by the entropy change, and we suppose this to be a common rule for most complex systems with uncharged ligands.

J. Bjerrum [1,p. 289] has previously shown that the consecutive formation constants of various metal ammonia systems in aqueous solutions of ammonium nitrate (until 5 M), and in a small interval around a standard temperature T_0 (*e.g.* $T = 298° \pm 5°$ K) can be expressed by formulae of the type:

$$\log K_n = \log K_n^\circ + \alpha\,(T_0 - T) + \beta \cdot C_{salt} \qquad (1)$$

where α and β are constants for the system in question for all values of n until the characteristic coordination number is reached. Introducing the heats and entropies for the uptake of the n'th ligand, we may put *:

$$\log K_n = \frac{\Delta S_n}{2.3\ R} - \frac{\Delta H_n}{2.3\ RT}\ ,$$

* In this paper quantities for the n'th step is denoted by K_n, ΔH_n, ΔS_n *etc.*, and the corresponding gross qualities by $K_{1\text{-}n}$, $\Delta H_{1\text{-}n}$, $\Delta S_{1\text{-}n}$ *etc.*

where

$$\Delta H_n = 2.3\, RTT_0\alpha \sim 2.3\, RT_0^2\alpha.$$

The validity of formula (1) shows that the heats of the successive steps are the same within the uncertainty of the experiment, and further that the heats do not vary with the salt concentration of the medium. The variation of log K_n (i) with the salt medium and (ii) with the number of ligands taken up in sequence is mainly to be found in the entropy: a very reasonable result which agrees well with the fact that the ratios between the consecutive constants have been shown to be mainly statistically determined [1].

Estimation of heats from temperature coefficients in systems with several consecutive constants demands a high precision of the measurements, and with some exceptions [2] this fact has led many authors to inconsistent results due to an overestimate of their data. In this paper we have, therefore, preferred to determine the heats by calorimetric titration of solutions in which the distribution of the complexes are known from affinity measurements. Examined were the nickel(II) and copper(II) systems with ethylenediamine (en) and trimethylenediamine (tn) * in 1 M KNO_3 and partly also in solutions free from neutral salt. Calvin [6] and later Schwarzenbach [4] suggested that complex formation with chelates gives a higher entropy than with simple ligands, and it was a special purpose of this investigation to study this effect for non-charged ligands forming 5- and 6-membered rings.

In this paper we discuss first the calorimetric measurements, then potentiometric determinations of the complexity constants, and finally the thermodynamic data.

EXPERIMENTAL

The calorimeter was a 500 ml nickelplated brass calorimeter with a glass stirrer driven by air pressure. The Beckman thermometer was adjusted for each five degrees and could be read with an error of 0.001 degree. The heater used in the measurements of the heat capacity was made of two nickelplated brass rods connected with ten 0.1 mm coiled coil tungsten wires. The current was measured by means of a precision ammeter, which could be read within an error of 0.2 %, the potential over the wires was measured on a Vernier potentiometer from Cambridge Instrument Co., and the time, with an uncertainty of about one half of a second, on a stop watch connected to the heater switch. A few experiments in which was measured the heat capacity of 1.2 M potassium nitrate solution as well as that of mixed solutions of potassium nitrate and barium (or nickel) nitrate, showed that it was permissible to exchange up to 10 % of the potassium nitrate with the equivalent amount of bivalent nitrate and still get the same heat capacity of the solution within one half of a per cent. The heat capacities of the mixed solutions could therefore be identified with the heat capacities of pure potassium nitrate solutions of same total nitrate normality. The data for potassium nitrate was taken from Randall and Rossini [7], and a curve giving the specific heat capacity at 25° C as function of moles KNO_3 per 1 000 g H_2O was used in evaluating the heat capacities of the mixed solutions in question. The heat capacity of the calorimeter + part of the stirrer + part of the thermometer was considered constant throughout the experiments and calculated to

* 1,3-Diaminopropane or trimethylenediamine, abbreviated to *tn* by Werner [3], has recently been called propylenediamine (pn) by Schwarzenbach [4]. This is a very unfortunate choice since propylenediamine has hitherto always been identified with 1,2-diaminopropane. Irving *et al.*[5] use the abbreviation dmp for 1,3-diaminopropane. This is a better choice, but it does not allow to distinguish between the two isomers.

be 20.1 cal/degree using values of the specific heat capacities for brass and glass given in the Landolt-Börnstein tables.

Procedure. The measurements, *e.g.* the reaction between nickel ion and ethylenediamine, were carried out with the same amount of ethylenediamine in almost all cases and varying amounts of nickel nitrate, in order to obtain the mole ratios en : Ni^{++} of 1 : 1, 1 : ½ and 1 : ⅓. The proper amounts of the stock solutions of the various salts and distilled water were weighed into the calorimeter to give a total amount of solution of 430 ml (calculated as the sum of the volumes of the stock solutions and the volume of the water added). The temperature was followed until the rate of increase remained constant for about 10 minutes, and in the next minute about 5 ml of the diamine stock solution was added through a hole in the cover of the calorimeter after which the temperature was followed until the rate of increase (or decrease) again was constant for 10 minutes. The diamine was added by means of a calibrated syringe pipette and its temperature was known within 0.01° C. The heat of dilution of the diamine stock solution was determined in an analogous experiment in which the nickel (or copper) nitrate was replaced normally with an equivalent amount of barium nitrate. Measurements at different concentrations showed that the heat of dilution for the salt medium used decreased almost linearly with increasing total concentration of nitrate ions (*cf.* Table 1 A).

Solutions. The nickel nitrate stock solution (0.5 M) was made from ANALAR reagent and the concentration in moles per 1 000 g of solution was determined by precipitation with dimethylglyoxime. The copper nitrate stock solution was made from Merck's copper(II) nitrate *p.a.*, and the concentration (moles per 1 000 g of solution) determined by thiocyanate titration according to Hagen [8]. Ethylenediamine (en) from Sharplet Chemical Inc. Phil. was distilled through a column, and the fraction taken at 117° C. was diluted 1 : 1 with distilled water. Trimethylenediamine (tn) from Sharplet Chemical Inc. Phil. was distilled in the same way and the fraction 134—137° C diluted 1 : 1 with distilled water. The molar concentration of the amine stock solutions (about 7 M) was determined by titration with standard acid solution using methyl orange as indicator. The densities of the stock solutions were determined in all cases in order to make a correlation of the different concentration units possible.

CALORIMETRIC DATA

The data were calculated from the usual calorimeter equation, with a correction for the amount of heat added with the diamine solution:

$$Q = (C + cW)\,(t_2 - t_1) - c_a w\,(t_a - t_1)$$

In this equation $C = 20.1$ cal/degree is the total heat capacity of the calorimeter, and W the total gram weight of the solution including the weight of the added amine. $t_2 - t_1$, the temperature increase in ° C during the reaction, was obtained by graphical extrapolation. The specific heat capacity of the reacting solution, c, was estimated graphically as mentioned above from the total nitrate normality per 1 000 g H_2O in the final solution. c_a, the specific heat capacity of the diamine solutions, was chosen as 0.8 cal/degree from a consideration of the values of the heat capacities of various other organic bases in aqueous solution at a similar concentration. t_a is the temperature, and w, the gram weight of the added amount of amine solution. All the calorimetric measurements were performed at 20—22° C.

Table 1 A gives the experimental details about the heats of reaction (Q) determined for two nickel nitrate solutions and the corresponding measurements of the heats of dilution (Q_{dil}) of ethylenediamine in barium nitrate containing solutions. The difference $Q - Q_{dil}$ for solutions of the same total nitrate normality should be the heat of the ammine formation. Ethylene-

Table 1 A. Detailed calorimetric data for the reaction of two nickel and three barium solutions of similar nitrate normalities with a fixed amount of ethylenediamine.

No.	moles of KNO_3	moles of $Ni(NO_3)_2$	moles of en	moles NO_3^- per 1 000 g H_2O	W	c	t_2-t_1	w	t_a-t_1	Q
3	0.5156	0.01788	0.03856	1.36	468.5	0.882	0.901	5.3	−1.15	395.3
4	0.5162	0.01130	0.03856	1.31	467.8	0.885	0.828	5.3	+0.54	357.2
		moles of $Ba(NO_3)_2$								Q_{dil}
	0.5153	0.04292	0.03856	1.47	473.7	0.873	0.083	5.3	−0.66	38.8
3′	0.4752	0.04309	0.03856	1.37	472.6	0.881	0.088	5.3	−0.57	40.8
4′	0.5156	0.01295	0.03856	1.32	468.7	0.884	0.099	5.3	+0.40	41.3

diamine and trimethylenediamine are somewhat dissociated in unbuffered solutions of non-complex forming salts, but according to measurements in the literature [9] their heats of dissociation are very small, and it has been found unnecessary to introduce a correction for these heats in our calculations.

To obtain the heats of formation of the mono complexes Ni tn^{++} and Cu en^{++}, it was necessary to avoid precipitation of the hydroxides. Therefore we buffered the solutions by adding some nitric acid and a little more than the equivalent amount of diamine to the calorimeter solution before the reaction. Then using a suitable amount of the base for the reaction it was possible to keep the concentration of the diammonium ions constant during the reaction. The diammonium salt was replaced with an equivalent amount of potassium nitrate in the corresponding determination of Q_{dil}.

A summary of all the calorimetric data is given in Table 1 B. In the experiments where nitric acid has been added, measurements of the hydrogen ion concentration ascertained that all the acid was neutralized by the base to form diammonium ions. Therefore, $\bar{n}^0$ and $\bar{n}$, the average number of diammine molecules bound per metal ion before and after reaction, respectively, could be directly deduced from the total amount of diamine after deduction of the amount bound as enH_2^{++} or tnH_2^{++}. In the unbuffered solutions $\bar{n}$ was taken normally as the ratio of moles of diamine to moles of nickel or copper ions in the solution. When $\bar{n}$ approaches 3 we have, especially in the nickel trimethylenediamine system, an appreciable amount of free base in the solution, and in these cases $\bar{n}$ was computed using our knowledge of the complexity constants. The last column in Table 1 B gives ΔH_a, the heat of formation per mole of diamine complexly bound to the metal ion, and it is seen that this quantity is nearly constant, independent of the composition of the solutions from which it is deduced. It is also seen that the heat of reaction is nearly independent of the salt medium, though it appears that ΔH_a is slightly higher in 1 M KNO_3-solutions than in solutions free from neutral salt.

The quantities we are especially interested in are the heats of the individual complexes. These were calculated from the measurements of solutions with *ca.* 1 M KNO_3. Denoting the heats of formation per mole of metal atom

Table 1 B. Summary of calorimetric data of the systems examined.

System No.	C_{KNO_3} (moles per liter)	moles of HNO_3	moles of $Me(NO_3)_2$	moles of amine before react.	moles of amine after react.	$\bar{n}^\circ$ before react.	$\bar{n}$ after react.	Q (cal.)	Q_{dil} (cal.)	$-\Delta H_a$ (kcal.)
Ni++, en										
1	1.0	0.0864	0.0432	0.0438	0.0823	0.014	0.905	388.3	39.2	9.07
2	1.0	0.0857	0.0429	0.0436	0.0821	0.016	0.914	387.2	39.2	9.04
3	1.2	0	0.0179	0	0.0386	0	2.16	395.3	40.8	9.18
4	1.2	0	0.0113	0	0.0386	0	3.00	357.2	41.3	9.32
5	1.2	0	0.0386	0	0.0386	0	1.00	391.2	39.6	9.11
5a*	1.2	0	0.0386	0.0386	0.0772	1.00	2.00	396.0	39.6	9.23
5b*	1.2	0	0.0386	0.0772	0.1157	2.00	2.98	398.2	39.6	9.48
6	0	0	0.0223	0	0.0449	0	2.01	449.0	54.9	8.78
7	0	0	0.0223	0	0.0449	0	2.01	454.0	54.9	8.89
8	0	0	0.0123	0	0.0449	0	3.00	393.8	56.4	9.14
9	0	0	0.0123	0	0.0449	0	3.00	389.4	56.4	9.02
Cu++, en										
1	1.0	0.0431	0.0434	0.0236	0.0621	0.046	0.933	542.9	40.8	13.04
2	1.0	0.0427	0.0433	0.0235	0.0620	0.048	0.938	538.5	40.8	12.93
3	1.1	0	0.0178	0	0.0392	0	2.00	494.7	42.3	12.71
4	1.1	0	0.0178	0	0.0392	0	2.00	494.6	42.3	12.71
5	0	0	0.0159	0	0.0449	0	2.00	451.8	55.0	12.48
6	0	0	0.0160	0	0.0449	0	2.00	447.8	55.0	12.28
Ni++, tn										
1	1.0	0.0428	0.0430	0.0222	0.0519	0.019	0.709	293.7	64.8	7.71
2	1.0	0.0428	0.0430	0.0222	0.0519	0.019	0.709	296.0	64.8	7.78
3	1.2	0	0.0221	0	0.0443	0	1.97	423.9	96.5	7.52
3a*	1.2	0	0.0221	0.0443	0.0886	1.97	2.56	183.6	96.5	6.68
4	1.1	0	0.0200	0	0.0443	0	2.09	409.2	97.7	7.45
5	1.1	0	0.0129	0	0.0443	0	2.35	318.5	97.7	7.28
Cu++, tn										
1	1.1	0	0.0180	0	0.0443	0	2.00	507.8	97.7	11.39
2	1.1	0	0.0180	0	0.0443	0	2.00	506.9	97.7	11.37

* The measurements are carried out with successive addition of the diamine solution.

by ΔH, and the gross heats for the complexes by ΔH_1, ΔH_{1-2}, *etc.*, we have for a system of three complexes:

$$\Delta H = (\alpha_1 - \alpha_1^\circ)\,\Delta H_1 + (\alpha_2 - \alpha_2^\circ)\,\Delta H_{1-2} + (\alpha_3 - \alpha_3^\circ)\,\Delta H_{1-3}$$

In this equation α_1, α_2 and α_3 denote the degrees of formation of the three complexes in the reaction mixture, and α_1°, α_2° and α_3° the same quantities in the starting solutions. Equations of this kind were combined in various ways in order to calculate the heats in question, which after all were only a little different from the total heats for all integral numbers of n. The α-quantities were calculated by means of the complexity constants to be discussed in the next section, and the computed heats are summarized in Table 1 C. The uncertainty in the heats is *ca.* $\pm$ 1 %, except for the values in the nickel trimethylenediamine system for which the uncertainty is about 2 %.

Table 1 C. Estimated heats of formation in kcal of the individual complexes in ca. 1 M KNO_3.

System	Combination of Nos.	$-\Delta H_1$	$-\Delta H_{1-2}$	$-\Delta H_{1-3}$
Ni++, en	1, 3, 4	9.03	18.23	27.96
	1, 5a, 5b	9.06	18.17	27.79
	2, 3, 5b	8.89	18.28	27.88
	2, 4, 5a	8.94	18.04	27.96
	3, 4, 5	9.09	18.25	27.96
	5, 5a, 5b	9.12	18.19	27.82
	Average	9.01	18.19	27.90
Cu++, en	1, 3	13.13	25.42	
	2, 4	12.93	25.41	
	Average	13.03	25.42	
Ni++, tn	(1,2), 3, 3a	7.77	15.05	21.70
	(1,2), 3, 5	7.77	15.06	20.94
	(1,2), 3a, 5	7.77	14.78	21.46
	(1,2), 3a, 4	7.77	15.01	21.67
	(1,2), 4, 5	7.77	15.11	20.93
	Average	7.77	15.00	21.34
Cu++, tn	Average		22.76	

POTENTIOMETRIC DETERMINATION OF COMPLEXITY CONSTANTS

The formation constants of the nickel(II) ethylenediamine system have been determined by various authors [1,10–14] at various salt concentrations and temperatures, but the results do not agree very well. In this work they were, therefore, determined again at 25° from glass electrode measurements under conditions similar to those used by J. Bjerrum and co-workers [15,16] for the zinc(II), cadmium(II) and copper(II) ethylenediamine systems, *i.e.* the following total concentrations $C_{Me++} = 0.1$ M, $C_{HNO_3} = 0.1$ M, $C_{KNO_3} =$ 1 M and 0.05 M $< C_{en} <$ 0.5 M. The techniques were the same as used by J. Bjerrum and Nielsen [16], and we are indebted to Mr. C. J. Ballhausen for making these measurements. A selection of the data are given in Table 2, in which column 5 gives $\bar{n}_{en}$, the average number of hydrogen ions bound to not complexed ethylenediamine, and column 7 the average number of ethylenediamine bound to nickel ions. The data were computed in the usual way from the values of — log[en] for $\bar{n} = 0.5$, 1.5 and 2.5. The following values for the consecutive formation constants were obtained:

$$\log K_1 = 7.51, \qquad \log K_2 = 6.35, \qquad \log K_3 = 4.42.$$

The data of Carlson, McReynolds and Verhoek [10] and of Hares [13] seem to be in rather good agreement with these values.

The first two formation constants of the nickel(II) and copper(II) trimethylenediamine systems have been determined by Hares [13] in 1 M KNO_3 at 0° and 30°. Irving *et al.*[5] also recently determined the constants in the last mentioned system in 0.1 M KCl at 25°. In this work all three of the consecutive formation constants in the nickel and both of the formation constants in the copper system were determined at 25° C and at the same total concentrations as used in the ethylenediamine systems. There were no diffi-

Table 2. Glass electrode measurements of diamine containing metal salt solutions at 25° C.

Ni^{++}, en: Calc. with $-\log K_{enH_2^{++}} = 7.49$, $-\log K_{enH^+} = 10.17$.
$C_{HNO_3} = 0.1004$ M, $C_{KNO_3} = 1.00$ M.

No.	$C_{Ni(NO_3)_2}$	C_{en}	$-\log [H^+]$	$\bar{n}_{en}$	$-\log [en]$	$\bar{n}$
1	0.0947	0.0870	5.601	1.988	7.762	0.386
2	0.0947	0.1170	5.836	1.980	7.304	0.701
3	0.0947	0.1281	5.912	1.978	7.144	0.817
4	0.0947	0.1678	6.175	1.954	6.622	1.229
5	0.0947	0.1904	6.301	1.938	6.374	1.462
6	0.0947	0.2073	6.421	1.922	6.128	1.640
7	0.0947	0.2882	7.226	1.651	4.613	2.402
8	0.0947	0.3156	7.447	1.527	4.229	2.599

Ni^{++}, tn: Calc. with $-\log K_{tnH_2^{++}} = 9.12$, $-\log K_{tnH^+} = 10.62$.
$C_{HNO_3} = 0.0994$ and 0.0990 M (No. 11), $C_{KNO_3} = 1.00$ M.

No.	$C_{Ni(NO_3)_2}$	C_{tn}	$-\log [H^+]$	$\bar{n}_{tn}$	$-\log [tn]$	$\bar{n}$
1	0.1002	0.0810	7.143	1.990	6.760	0.310
2	0.1000	0.1122	7.427	1.980	6.194	0.620
3	0.1000	0.2042	8.359	1.852	4.363	1.508
4	0.0997	0.2338	8.647	1.745	3.817	1.778
5	0.1004	0.3010	9.671	1.139	2.091	2.129
6	0.1000	0.3527	10.163	0.820	1.531	2.315
7	0.1002	0.4116	10.463	0.626	1.197	2.523
	$C_{HNO_3} = 0.01992$ M, $C_{KNO_3} = 1.08$ M, $C_{BaCl_2} = 0.08$ M.					
8	0.02048	0.02203	7.734	1.961	6.283	0.579
9	0.02062	0.04296	8.736	1.707	4.351	1.521

Cu^{++}, tn: Calc. with $-\log K_{tnH_2^{++}} = 9.12$, $-\log K_{tnH^+} = 10.62$.
$C_{HNO_3} = 0.01992$ M, $C_{KNO_3} = 1.08$ M, $C_{BaCl_2} = 0.08$ M.

No.	$C_{Cu(NO_3)_2}$	C_{tn}	$-\log [H^+]$	$\bar{n}_{tn}$	$-\log [tn]$	$\bar{n}$
1	0.02111	0.04326	7.328	1.984	7.089	1.574
2	0.02119	0.04570	7.464	1.978	6.818	1.681

culties in preparing nickel trimethylenediamine solutions of this composition, but the corresponding copper solutions separated basic precipitates unless $\bar{n} \gtrsim 1.5$. For this reason the gross complexity constant K_{1-2} was determined with copper amalgam electrode in solutions with excess of diamine.

Table 3 gives some glass electrode measurements of the acid-base constants of trimethylenediamine in solutions with 0.1 M HNO_3, 0.1 M $BaCl_2$ and 1 M KNO_3. We are indebted to Mrs. S. Refn for making these measurements. The true hydrogen ion concentration was determined by measuring against a standard acid solution of the composition: $C_{HNO_3} = 0.005$ M, $C_{BaCl_2} = 0.1$ M, $C_{KNO_3} = 1.1$ M. For low values of $\bar{n}_{tn} = C_{HNO_3}/C_{tn}$ the solutions were so alkaline that the glass electrode might show deviations from the reversible hydrogen electrode. Therefore the product of the acid dissociation constants of the diammonium ion was determined from the interpolated hydrogen ion concentration ($= 10^{-9.87}$) for $\bar{n}_{tn} = 1$:

$$K_{tnH_2^{++}} K_{tnH^+} = [H^+]^2_{\bar{n}=1} = 10^{-19.74}.$$

Table 3. Acid-base constants of trimethylenediamine at 25° C.
C_{KNO_3} = 1.00 M, $BaCl_2$ = 0.100 M.

No.	C_{HNO_3}	C_{tn}	$-\log [H^+]$	$\bar{n}_{tn}$	$-\log K_{tnH^+}$
1	0.0994	0.1051	9.965	0.946	
2	0.0994	0.1012	9.897	0.982	
3	0.0994	0.09282	9.759	1.071	
			9.87 (int.)	1.00	
4	0.0994	0.06765	9.134	1.475	9.134
5	0.0994	0.06712	9.114	1.481	9.121
6	0.0994	0.06398	8.997	1.554	9.119

$K_{tnH_2^{++}}$ was determined from the formula

$$K_{tnH_2^{++}} = \frac{(2-\bar{n})\,[H^+]^2 - \bar{n} K_{tnH_2^{++}} K_{tnH^+}}{(\bar{n}-1)\,[H^+]}$$

in the buffer range for this constant. In average was found $-\log K_{tnH_2^{++}} = 9.12$, $-\log K_{tnH^+} = 10.62$. From Hares' [13] data at 20° and 30° C one computes the values 9.10 and 10.70, respectively, in 1 M KNO_3 at 25° C.

A selection of the measurements made on nickel and copper trimethylenediamine solutions is given in Table 2. In order to vary the concentration of the complex-forming metal ion without changing the salt medium the 0.1 molar concentration of nickel (or copper) nitrate was in some cases (see Table 2) partly exchanged with an equivalent concentration of barium chloride, and the 0.1 molar nitric acid correspondingly with potassium nitrate. The consecutive constants in the nickel system were obtained from the values of $-\log [tn]$ for $\bar{n} = 0.5$, 1.5, and 2.5, which were determined graphically to be 6.40, 4.38, and 1.23, respectively. Because of the very slight overlap of the three steps, these values differ only a little from the computed formation constants:

$$\log K_1 = 6.39, \qquad \log K_2 = 4.39, \qquad \log K_3 = 1.23.$$

They do not agree very well with Hares' [13] values for the first two formation constants. From his data we get: $\log K_1 = 5.58$, $\log K_2 = 3.52$ in 1 M KNO_3 at 25° C.

The gross complexity constant in the copper trimethylenediamine system was determined by measuring with the copper amalgam electrode a series of copper(II) trimethylenediamine solutions with excess of diamine. A 1 N calomel electrode was used as reference electrode. The cells examined and a selection of the measurements made are summarized in Table 4. The measurements were made in an atmosphere of nitrogen applying the same technique as previously used by Bjerrum and Nielsen [16] in the determination of the gross complexity constant in the copper(II) ethylenediamine system. From the directly measured potentials E the normal potential copper amalgam-copper(II) ion (relative to the calomel electrode) is calculated by means of the expression

$$E = E^\circ_{Cu,Cu^{++}} + \frac{RT}{2F} \ln [Cu^{++}]$$

Table 4. The normal oxidation potentials Cu,Hg → Cu^{++} and Cu,Hg→ $Cu\,tn_2^{++}$ (relative to the normal calomel electrode) in 1 M KNO_3 at 25° C from measurements of the cells:

No. 1: Cu,Hg | $C_{Cu(NO_3)_2}$, C_{HNO_3}, 1.10 M KNO_3 | KCl sat. | 1 M KCl, Hg_2Cl_2 | Hg

Nos. 2–4: Cu,Hg | $C_{Cu(NO_3)_2}$, C_{tn}, C_{HNO_3}, 1.0 M KNO_3 | KCl sat. | 1 M KCl, Hg_2Cl_2 | Hg

No.	$C_{\overline{Cu(NO_3)_2}}$	C_{HNO_3}	C_{tn}	[tn]	E	$\frac{RT}{2F}\ln C_{Cu^{++}}$	$E°_{Cu,Cu^{++}}$
1	0.0998	0.0024	0	0	+ 0.0190	− 0.0296	+ 0.0486
						$\frac{RT}{2F}\ln\frac{C_{Cutn_2^{++}}}{[tn]^2}$	$E°_{Cu,Cutn_2^{++}}$
2	0.0997	0.0500	0.3703	0.1208	− 0.4345	+ 0.0247	− 0.4592
3	0.0996	0.0500	0.3906	0.1414	− 0.4384	+ 0.0206	− 0.4591
4	0.0999	0.0501	0.4113	0.1614	− 0.4439	+ 0.0173	− 0.4612

and the normal potential copper amalgam-bis(trimethylenediamine) copper (II) ion by means of the expression

$$E = E°_{Cu,Cutn_2^{++}} + \frac{RT}{2F}\ln\frac{[Cu\,tn_2^{++}]}{[tn]^2}$$

From the results of Randles' [17] investigation of the equilibrium between copper(I) and copper(II) ions in the presence of trimethylenediamine we estimate that for a copper concentration of about 0.1 M and a concentration of free diamine less than 0.2 M, it seems reasonable to neglect any disturbing influence of copper(I) ions. The copper(II) ion concentration to be used in the calculation was assumed to be the same as the total copper(II) concentration in the acid solution. In the solutions with trimethylenediamine it was assumed that the copper was present solely as the bis(trimethylenediamine) complex and that the concentration of the monoammonium ion tnH^+ was equal to the nitric acid concentration added.

The computed value for the normal potential of the copper-trimethylenediamine system shows a slight tendency to fall with increasing concentration of free diamine. This can perhaps be explained in the same manner as the observed fall in the normal potential of the corresponding copper-ethylenediamine system [16]. If the values of the normal potentials of $E°_{Cu,Cu^{++}}$ and $E°_{Cu,Cutn_2^{++}}$ from solutions 1 and 2 are combined, we get for the gross constant in question

$$\log K_{1-2} = \frac{0.0486 + 0.4592}{0.02957} = 17.17.$$

Since $K_1 \gg K_2$, values for the second consecutive constant can be directly obtained from the measurements in Table 2. From solutions 1 and 2 are estimated $\log K_2 = 7.22$ and 7.15, respectively. As an average we therefore get for the consecutive constants $\log K_1 = 9.98$, $\log K_2 = 7.19$. These values agree fairly well with Hares' [13] values: $\log K_1 = 9.79$, $\log K_2 = 7.16$ in 1 M KNO_3 at 25° C. Irving *et al.*[5] estimate similar values in 0.1 M KCl, but find

Table 5. Thermodynamic data for some nickel and copper (II) amine systems at 25° C.

System	log K ($\mu\sim1$)	$-\Delta G$ kcal.	$-\Delta G'$ kcal.	$-\Delta H$ kcal.	ΔS e.u.	$\Delta S'$ e.u.	Difference between consecutive ΔS values	Statistical effect
Ni^{++} + 3en	18.28	24.90	31.98	27.90	−10.1	13.6		
n = 1	7.51	10.23	12.59	9.01	4.1	12.0		
n = 2	6.35	8.65	11.01	9.18	− 1.8	6.1	5.9	3.12
n = 3	4.42	6.02	8.38	9.71	−12.4	− 4.5	10.6	4.44
Ni^{++} + 3tn	12.01	16.36	23.44	21.3	−16.6	7.1		
n = 1	6.39	8.69	11.05	7.8	3.0	10.9		
n = 2	4.39	5.98	8.34	7.2	− 4.1	3.8	7.1	3.12
n = 3	1.23	1.68	4.04	6.3	−15.5	− 7.6	11.4	4.44
Ni^{++} + $6NH_3$	8.61	11.73	25.89	21.0	−31.1	16.3		
n = 1	2.77	3.77	6.13	3.5	0.9	8.8		
n = 2	2.22	3.02	5.38	3.5	− 1.6	6.3	2.5	1.74
n = 3	1.71	2.33	4.69	3.5	− 3.9	4.0	2.3	1.25
n = 4	1.17	1.59	3.95	3.5	− 6.4	1.5	2.5	1.14
n = 5	0.73	0.99	3.35	3.5	− 8.4	− 0.5	2.0	1.25
n = 6	0.01	0.01	2.37	3.5	−11.7	− 3.8	3.3	1.74
Cu^{++} + 2en	20.03	27.28	32.00	25.4	6.3	22.1		
n = 1	10.72	14.60	16.96	13.0	5.4	13.3		
n = 2	9.31	12.68	15.04	12.4	0.9	8.8	4.5	4.13
Cu^{++} + 2tn	17.17	23.39	28.11	22.8	2.0	17.8		
n = 1	9.98	13.59	15.95					
n = 2	7.19	9.79	12.15					
Cu^{++} + $4NH_3$	12.63	17.20	26.64	20.0	− 9.4	22.2		
n = 1	4.14	5.64	8.00	5.0	2.1	10.0		
n = 2	3.49	4.75	7.11	5.0	− 0.8	7.1	2.9	1.95
n = 3	2.88	3.92	6.28	5.0	− 3.6	4.3	2.8	1.61
n = 4	2.12	2.89	5.25	5.0	− 7.1	0.8	3.5	1.95
n = 5	− 0.55	− 0.75	1.61	3.2	−13.3	− 5.4		

for concentrations of free diamine higher than $10^{-4.5}$ that $\bar{n}$ rises slightly above two. Irving *et al.* mention that a corresponding effect is not found in the spectra. This seems to show that not more than 4 nitrogen atoms are coordinated to the copper.

DISCUSSION OF THERMODYNAMIC DATA

The complexity constants and heats determined for the nickel and copper diamine systems are collected in Table 5. Davies, Singer and Staveley [9] recently measured the heat of formation of the ethylenediamine complexes of

nickel, copper, zinc, and cadmium by a more accurate calorimeter setup than used in our investigation, but as they did not measure below $\bar{n}$ equals 1.9, their heats for the first complex cannot be very accurate as they state themselves. For the other complexes they find the following figures in fair agreement with our determinations

	Ni	Cu	Zn	Cd
$-\Delta H_{1-2}$	17.25	25.16	11.45	13.33
$-\Delta H_{1-3}$	28.01		18.46	19.70

It will be seen that Davies *et al.* also find roughly the value 3 : 2 for the ratio of the heats of the 1-3 and 1-2 complexes in the zinc and cadmium systems. In the copper ethylenediamine system Spike and Parry [2] using the temperature coefficient method found the values $-\Delta H_1 = 14.6$, and $-\Delta H_{1-2} = 28.4$ to be compared with the value $-\Delta H_{1-2} = 26.1$ of Bjerrum and Nielsen [16]. Also for other amine systems Spike and Parry [2] obtained similar results in fair agreement with the assumption that the heats of successive steps are the same within the uncertainty of the experiment. There is, therefore, some justification for Bjerrum's use of equation (1), and we consider this to be a common rule for all normal complex systems with dipole ligands: if by normal is understood systems free from steric strain in which no change in configuration and total spin quantum number occurs [18,19]. In the nickel trimethylenediamine system ΔH_3 seems to be somewhat smaller than ΔH_1 and ΔH_2. This might be due to the greater error in the determination of the heat for the third complex in this case, but paying due regard to the great spreading of the complexity constants it may also be caused by the steric strain in the system (*cf.* Ref. 1, p. 91). However, it is noteworthy that the relatively small affinity to the 3rd ethylenediamine in the zinc and cadmium systems does not show itself in the heats determined by Davies *et al.*[9]

In Table 5 are the values found for the diamine complexes compared with corresponding data for the ammonia complexes. The complexity constants are J. Bjerrum's values in 1 M NH_4NO_3 at 25°, and the heats are taken from the literature. The value for $-\Delta H_{1-6} = 21$ in the nickel system is estimated from Fyfe's calorimetric measurements [20] considering that the complex formation is not completed for $[NH_3] \sim 0.5$ M. The values for ΔH_{1-4} and ΔH_5 in the copper ammonia system are computed from the thermochemical measurements of Bouzat [21] (*cf.* Ref. 22, p. 18). To obtain the heats of the single steps we have divided the total heat by the number of uniformly bound ligands N using the approximation contained in the equation (1) about the equality of the ΔH_n values.

The large chelate effect of ethylenediamine relative to ammonia defined by Bjerrum and Nielsen [16] as

$$-\Delta G\,(\tfrac{1}{2}\,\mathrm{en}) + \Delta G\,(NH_3) = \frac{RT}{N}\left(\frac{1}{2}\ln K_{1-N}\,(\mathrm{en}) - \ln K_{1-N}\,(NH_3)\right) \quad (2)$$

and by Schwarzenbach [4] as

$$\mathrm{Chel} = \log K_1\,(\mathrm{en}) - \log K_{1-2}\,(NH_3) \quad (3)$$

has been discussed by several authors. Schwarzenbach [4] has discussed the mechanism of chelation and presented an explanation of the effect. As Calvin and Bailes [6] he considers it to be an entropy effect. Spike and Parry [3] has shown that this is very nearly the case in the zinc and cadmium systems as different from the copper and nickel systems, for which the chelate effect is partly an enthalpy effect. The data in Table 5 show directly that, *e.g.*, $-\Delta H_1$ (en) is considerably higher than $-\Delta H_{1-2}$ (NH_3) in these systems. In this connection it is interesting that the chelate effect is relatively the highest for the transition group elements as first pointed out by Bjerrum and Nielsen (*cf.* Fig. 1 in Ref. 16). The loss in enthalpy by chelation of transition elements is therefore perhaps related to the fact that the crystal field stabilization [23] is higher for the ethylenediamine than for the ammonia complexes. A simple calculation shows that this stabilization amounts to about 1 kcal in the nickel systems or about half of the loss in enthalpy by exchange of two ammonia molecules with one ethylenediamine.

For a complex reaction of the type:

$$MA_{n-1} + A = MA_n + \text{water}$$

the formation constant: $K_n = \dfrac{[MA_n]}{[MA_{n-1}]\,[A]}$

as well as the free energy: $\Delta G_n = -RT \ln K_n$,

and the entropy: $$\Delta S_n = \frac{1}{T}(\Delta H_n - \Delta G_n)$$

is dependent on the standard states of the reactants. To escape this dependence on the standard states, Bjerrum [24] introduced the water concentration in the equilibrium constant and used the quantity $\frac{1}{Z} \log K_{1-n} + \log 55$, where Z is the characteristic coordination number, as a more correct measure for the affinity than $\frac{1}{Z} \log K_{1-n}$. Adamson [25] who takes another starting point introduces the mole fraction instead of molar concentrations in K_n. In the simple case for $Z = N$ the two treatments lead to the same result, but in case of chelation, *e.g.* for $Z = 2\ N$, the two treatments give different results as Bjerrum introduces no correction for the asymmetry of *e.g.* the reaction

$$MA_N + \frac{N}{2}\,\text{en} = M\,\text{en}_{\frac{N}{2}} + NA.$$

Following Adamson, we have corrected the usual values for ΔG (in kcal) and ΔS (in e.u.) by means of the formulae:

$$\Delta G' = \Delta G + \Delta\nu\, RT \ln 55.5 = \Delta G + 2.36\Delta\nu$$
$$\Delta S' = \Delta S - \Delta\nu\, R \ln 55.5 = \Delta S - 7.9\Delta\nu$$

where $\Delta\nu$ denotes the moles of products minus those of reactants, exclusive of solvent.

The data given in Table 5 show that the chelate effect according to (2) and (3) in the main is an arbitrary dilution effect which nearly vanishes when the corrected free energies are considered. This result, of course, does not interfere with the statements of Schwarzenbach, but has something to do with the fact that the entropy changes for reactions between reactants in pure states usually are small. The corrected entropy $\Delta S'_{1-6} = 16$ e.u. in the nickel ammonia system is seen to be only little different from the corresponding entropy $\Delta S'_{1-3} = 14$ e.u. in the nickel ethylenediamine system, and is distinctly higher than $\Delta S'_{1-3} = 7$ e.u. in the nickel trimethylenediamine system. In the corresponding copper systems $\Delta S'_{1-4}(NH_3) = 22$ e.u. is equal to $\Delta S'_{1-2}$ (en), and $\Delta S'_{1-2}$ (tn) = 18 e.u. is also here somewhat smaller.

Latimer and Jolly [26,27] have estimated the heat of reaction for the successive steps in the formation of the aluminium fluoride complexes. They found that the ΔS values decrease with the stepwise formation of the complexes and assume this to be due to a charge effect, which can be calculated by deducting the constant value of the replacement entropy 15.6 e.u. from the ΔS values. This replacement entropy should be due to the reaction:

$$H_2O \text{ (bound)} + F^- \text{ (aq.)} = H_2O \text{ (liq.)} + F^- \text{ (bound)}.$$

In the same paper they suggest the value 5.5 e.u. for the replacement

$$H_2O \text{ (bound)} + NH_3 \text{ (aq.)} = H_2O \text{ (liq.)} + NH_3 \text{ (bound)}$$

and argue that this is the principal effect on ΔS in complex formation with this ligand, as it is uncharged. Contrary to this suggestion the data in Table 5 show that also the ΔS values for the stepwise formation of the complexes with uncharged ligands decrease and in much the same way as for the charged ligand F^-.

Different empirical equations for calculation of the partial molar entropy $\overline{S}^\circ$ for complex ions have been put forward recently by Cobble [28], and George, Hanania and Irvine [29]. Cobble calculated the $\overline{S}^\circ$ value for the ion $Ni(en)_3^{++}$ in two different ways: (i) an entirely empirical value 163 e.u. and (ii) a partly empirical value 154 e.u. from the sum $\overline{S}^\circ_{Ni++} + 3\,\overline{S}^\circ_{en} + \Delta S_{1-3}$ for the reaction

$$Ni^{++} + 3\,en = Ni\,(en)_3^{++}$$

For ΔS_{1-3} they used the value + 41 e.u. determined by Basolo and Murmann [12]. If our value —10 e.u. is used for the same calculation, we get $\overline{S}^\circ$ equal to 103 e.u., which is much lower than the empirical value 163 e.u.

The value of the equation proposed by George *et al.*[29]

$$\overline{S}^\circ = A - Bz + CN$$

where A, B and C are numerical constants for the ligand in question, while N is the total number of ligands in the complex ion and z is the charge, is that the ΔS values of the successive steps in the formation of the complexes are zero when corrected by the statistical factors. This is in agreement with our results within the 3.4 e.u. mentioned by the authors except for the last step in the nickel diamine systems, but more experimental work is necessary before

much can be said about the values of the constants A and B for the ethylenediamine and trimethylenediamine complexes.

We have calculated the difference in ΔS between two successive steps in the complex formation and comparing these differences with the statistical effects [1,27] in Table 5 it will be noticed that they are very much alike in behavior although the differences seem to be twice as high as the statistical effects.

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Electrolytic Constants and Solubilites of Humulinic Acid, Humulone, and Lupulone

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The dissociation constants, solubility products and intrinsic solubilities of humulinic acid, humulone, and lupulone have been determined at 25 and 40° C. The solubility and its pH-dependence including extrapolated values for 0 and 100° C are shown in a diagram.

The bitter acids of hops represent a complex mixture containing a few well-defined components, humulone, lupulone, and some recently discovered homologues. Although much work has been done on these substances, their electrolytic constants have hitherto not been determined *. The solubility of humulone and lupulone at some pH-values was determined by Wöllmer [2] by titrating buffer solutions with alcoholic solutions of these substances until turbidity occurred. More refined methods have not been tried.

In the present investigation the dissociation constants and solubility products of humulone and lupulone at 25 and 40° C have been determined by means of a titration method proposed by Back and Steenberg [3]. Determinations have also been made on humulinic acid which, though only feebly bitter, is closely related to the derivatives of humulone, so far ill-defined, which constitute the chief bitter substances of the finished beer [4,5]. From these constants it is possible to calculate the solubility at all pH-values at 25 and 40° C and also extrapolated values for 0 and 100° C. At higher temperatures direct determination is not possible because of the low stability of humulone.

EXPERIMENTAL

Materials. The bitter acids were isolated from an ethereal extract of lupulin. Humulone was obtained by precipitating lead humulate from a solution of the lupulin extract in methanol, which was then decomposed in an ethereal solution by hydrochloric acid. The crude product was purified from oxidation products and the humulone homologues, adhumulone and cohumulone, by precipitating it twice more by lead acetate from methanol

* *Cf.*, however, the two-phase titrations of Hedlund and Steninger [1].

and once by *o*-phenylenediamine from benzene. The *o*-phenylenediamine complex was recrystallized from benzene until its melting point reached 117.5° C, the value stated for the pure humulone complex [6] (adhumulone complex [7] m. p. 98° C, cohumulone complex [8] m. p. 93° C). Pure humulone m. p. 65° C was then obtained by treating the complex with hydrochloric acid.

Lupulone was crystallized from the residue of the lupulin extract dissolved in petroleum ether. It was purified by recrystallization alternately from petroleum ether and methanol-water mixtures. The finished product had m. p. 94° C.

Humulinic acid (m. p. 91° C) was prepared by treating humulone with alcoholic alkali and recrystallizing the product from ethanol as described by Wöllmer [8].

As far as possible all operations were performed under oxygen-free nitrogen and the products were stored in sealed nitrogen-filled ampullae.

Procedure. The titration was carried out in a thermostated, closed vessel equipped with a magnetic stirrer in an atmosphere of oxygen-free nitrogen to prevent the entrance of carbon dioxide and oxygen. The pH-value was measured with standard glass and calomel electrodes and an electronic pH-meter.

The concentration of the solution to be titrated cannot be chosen arbitrarily, but is determined to a certain extent by the dissociation constants and solubility products [3]. In this case there were used a 15 mM solution of humulinic acid and 2.5 mM solutions of humulone and lupulone. In the latter case the concentration was the highest one attainable.

As an extra check of the purity of the products used titrations were carried out on samples from more than one batch of each of the different compounds.

The procedure and the calculations have been described in detail by Back and Steenberg [3].

ELECTROLYTIC CONSTANTS

Table 1 gives the mean values of the thermodynamic solubility products, dissociation constants and intrinsic solubilities of humulinic acid, humulone, and lupulone at 25 and 40° C. The figures are supposed to be accurate within $\pm$ 0.10.

For comparison are included the figures obtained from Wöllmer's solubility values using eqn. 1 given below. If Wöllmer's values refer to room temperature the agreement with my values is not too good. The values for the intrinsic solubilities and thus for the solubility products also may have become too high in Wöllmer's experiments due to supersaturation. During the present investigation a tendency to supersaturation at the flocculation point was easy to observe, especially for lupulone. The values obtained in this region were therefore omitted from the calculations.

Table 1. Electrolytic constants for humulinic acid, humulone, and lupulone.

	Temp.	pK_S	pK_A	ps_{HA}
Humulinic acid:				
Present author	25° C	5.64	2.35	3.29
	40° C	5.51	2.43	3.08
Humulone:				
Present author	25° C	9.00	4.22	4.78
	40° C	8.86	4.26	4.60
Wöllmer	not given	8.7	4.0	4.7
Lupulone:				
Present author	25° C	11.69	6.18	5.51
	40° C	11.65	6.30	5.35
Wöllmer	not given	10.9	6.2	4.7

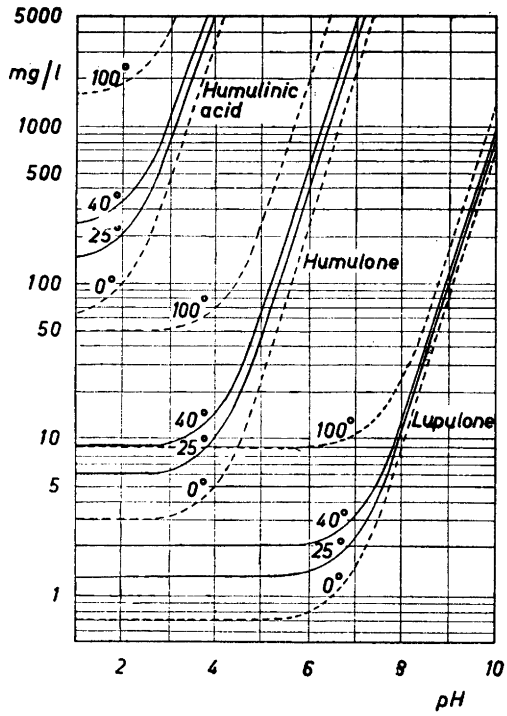

Fig. 1. Solubilities of humulinic acid, humulone, and lupulone.

SOLUBILITY DIAGRAM

The solubility s_{tot} of a slightly soluble acid HA is known to depend on the intrinsic solubility s_{HA}, the dissociation constant K_A, the solubility product K_S and the hydrogen ion activity $a_{H_3O^+}$, according to the equation

$$s_{tot} = s_{HA} + \frac{K_S}{a_{H_3O^+}\,\gamma_{A^-}} = s_{HA}\,(1 + \frac{K_A}{a_{H_3O^+}\gamma_{A^-}}) \qquad (1)$$

where

$$K_S = K_A \cdot s_{HA}$$

The solubility can thus be computed for any pH-value at 25 and 40° C using the constants given in Table 1.

The pH- and temperature dependence of the solubility may be visualized by a diagram of the Bjerrum type. If log s_{tot} is plotted against pH we get a curve of a simple form which, as is readily seen from eqn. (1), passes point (2 s_{HA}, pK_S) and has the two straight lines

$$\log s_{tot} = \log s_{HA} \qquad (\mathrm{pH} \ll \mathrm{p}K_A)$$

and

$$\log s_{tot} = \mathrm{pH} + \log K_A s_{HA} \qquad (\mathrm{pH} \gg \mathrm{p}K_A)$$

intersecting in point (s_{HA}, $p\mathrm{K}_A$) as asymptotes.

Fig. 1 gives the solubility curves for 25 and 40° C, as well as approximate curves for 0 and 100° C, obtained from values which have been calculated by extrapolation using the Clapeyron equation.

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S-Benzylthiuronium Salts

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171 S-Benzylthiuronium salts were prepared and were, if possible, titrated with perchloric acid. Melting points and a brief discussion on the influence of functional groups on the titration are given.

In the course of an investigation on the use of S-benzylthiuronium salts as derivatives of organic acids we have prepared a number of such salts which have not been described before.

Melting points and analyses of the derivatives are listed in the following tables, Table 1 giving the carboxylic acid derivatives and Table 2 derivatives of sulfonic acids. Melting points are uncorrected, they were measured in a paraffin-bath, the rate of heating being 2°/min.

Besides we have prepared a large number of already described salts [2,3,6,7 etc.], and as a rule we were able to reproduce the melting points given by these authors. In a few cases, when the melting points found by us deviated 10° or more from those reported by others, we have included the corresponding acid in Tables 1 or 2. However, as mentioned by Berger [2], the melting points of S-benzylthiuronium salts depend to a great extend on the rate of heating, and are, as such, not of much use for identification purposes.

Salts of xanthic acids (ROCSSH) have been prepared in two cases (Table 1); several others were synthesized, but could not be purified to give correct analysis. A salt of picric acid has been prepared (Table 1), whereas salts of dinitrophenols, 5-aminotetrazol and barbituric acids were not obtained in a sufficient degree of purity. We were unable to obtain salts from alifatic amino acids. Alifatic hydroxy acids have given salts in all cases tried: citric acid, however, gave a mixture of the normal and the secundary salts, which we were unable to separate.

EXPERIMENTAL

The derivatives of the monobasic acids were prepared according to Veibel *et al.*[6,7]: 10 m.equiv. of the acid is dissolved in 10 ml 1 N NaOH, the solution made slightly acidic (methyl red) or, if the acid is insoluble, as weakly alkaline as possible; if the sodium salt of the acid is insoluble it may be necessary to add more water. To the hot solution of the acid is added a hot solution of benzylthiuronium chloride (2 g, 10 m.equiv.), in 10 ml of

Table 1.

Acid	m. p.	equiv. weight	
		calc.	found
Acetic acid, allyl-	155—158	266	269
Acetic acid, bromo-	144—145	305	303
Acetic acid, dibenzyl-	138—139	407	402
Acetic acid, ethylxantogen-	149—150	347	345
Acetic acid, 1-naphthyl-	162—163	352	354
Acetic acid, phenyl-	165—166	302	300
Acetic acid, *n*-propylxantogen-	141—142	361	361
Acetic acid, thiol-	140—141	242	240
Acetic acid, triphenyl-	176—177	455	455
Acetoacetic acid, 2-benzeneazo-	175—176	372	370
Acetylenedicarboxylic acid, disalt,	160	223	226
Acrylic acid, 3-benzoyl-	127—129	342	346
Acrylic acid, 2-cyano-3-phenyl-	173	339	335
Acrylic acid, 3(2-furyl)-	170—171	304	308
Acrylic acid, 2-methyl-	156	252	249
Anthranilic acid, N-phenyl-	152—153	380	375
Azobenzene, 2,3,2′,3′-tetracarbonic acid, tetrasalt,	194	256	257
p-Benzenediacetic acid, 2,5-dihydroxy-, disalt,	199—200	279	280
Benzilic acid,	140	395	392
Benzoic acid, 2-acetoxy-5-nitro-	163—165	391	386
Benzoic acid, 3-amino-	160—161	303	302 (B)
Benzoic acid, 2-amino-5-chloro-	153—154	12.4 %	12.6 % (A)
Benzoic acid, 2-benzoyl-	177—178	393	388
Benzoic acid, 4-bromo-	195—196	367	365
Benzoic acid, 2-chloro-	165—166	323	319
Benzoic acid, 3-chloro-	164—165	323	319 (H)
Benzoic acid, 4-chloro-	190	323	321
Benzoic acid, 2-(4-chlorobenzoyl)-	165—166	427	422
Benzoic acid, 2,4-dichloro-	163—165	357	357
Benzoic acid, 3,4-dichloro-	168—169	357	353
Benzoic acid, 2,4-diethoxy-	145—146	377	374
Benzoic acid, 2,4-dihydroxy-	165—166	320	316
Benzoic acid, 2,4-dihydroxy-5-bromo-	170—171	399	395
Benzoic acid, 3,4-dinitro-	151—152	378	374
Benzoic acid, 3,5-dinitro-	178	378	373
Benzoic acid, 2-formyl-	121—122	316	320
Benzoic acid, 3-hydroxy-	161—162	304	304
Benzoic acid, 2-hydroxy-5-acetyl-	134—136	346	343
Benzoic acid, 2-(4-hydroxybenzoyl)-	195—196	408	403
Benzoic acid, 2-hydroxy-5-bromo-	183—184	383	385
Benzoic acid, 2-hydroxy-3,5-dichloro-	158—159	373	378
Benzoic acid, 2-hydroxy-3,5-diiodo-	103—107	556	562
Benzoic acid, 2-hydroxy-3,5-di*iso*propyl-	167—168	389	386
Benzoic acid, 2-hydroxy-5-iodo-	197—198	430	428
Benzoic acid, 2-hydroxy-5-nitro-	171—172	349	345
Benzoic acid, 2-iodo-	162—163	414	412
Benzoic acid, 3-iodo-	178—179	414	418
Benzoic acid, 2-methoxy-	163—164	318	323
Benzoic acid, 3-methoxy-	176	318	318
Benzoic acid, 3-nitro-4-methyl-	167—168	347	350

Table 1. continued.

Acid	m. p.	equiv. weight	
		calc.	found
Benzoic acid, 2-propionyl-	135	344	346
Benzoic acid, 2-(4-toluyl)-	171—172	407	400
Benzoic acid, 3,4,5-trimethoxy-	162—164	378	383
n-Butyric acid, 2-bromo-	146—147	333	335
n-Butyric acid, 2-bromo-3-methyl-	151—152	347	345
n-Butyric acid, 2-chloro-	166—167	289	285
n-Butyric acid, 4-*cyclo*hexyl-	154—155	337	335
n-Butyric acid, 2-ethyl-	133—134	282	280
d-Camphoric acid, disalt	137—138	266	264
Cinnamic acid, α-acetamino-	182—183	372	367
Cinnamic acid, α-methyl-	130—131	328	333
Cinnamic acid, 4-methyl-	175—176	328	325
Cinnamic acid, 3-nitro-	158—159	359	354
Cinnamic acid, 4-nitro-	208—209	359	354
Cinnamic acid, α-phenoxy-	177—178	407	402
Coumarin, 3-carboxylic acid,	167	356	359
Coumaric acid, O-methyl-	147—148	344	346
Coumarinic acid, O-methyl-	146—147	344	340
*Cyclo*hexane carboxylic acid,	165—166	294	292 (C)
Decanoic acid,	148—149	339	338
2,2′-Diphenic acid, monosalt,	174—176	409	412
Glutaric acid, disalt,	152—153	232	230
Glycine, N-acetyl-	166—167	283	280
Glycine, N-carbobenzyloxy-	139—140	375	374
10-Hendecenoic acid,	145—146	351	353
Hexanoic acid, 6-benzoylamino-	151—152	402	397
Hexanoic acid, 2-butyl-2-cyano-	150—151	364	362
Hexanoic acid, 2-ethyl-	129—130	311	315
Hippuric acid,	158—159	345	340
Hydrocinnamic acid, α-benzyl-α-cyano-	112—113	432	436
Levulinic acid,	133—134	282	279
Maleic acid, 2-chloro-, monosalt,	186—187	317	318
Malonic acid, allyl-, disalt,	147—153	238	235
Malonic acid, allylethyl-, disalt,	141—144	252	252
Malonic acid, benzyl-, disalt,	161—162	263	264
Malonic acid, *n*-butyl-, disalt,	132—135	246	246
Malonic acid, butylethyl-, monosalt	137—138	355	351
Malonic acid, diethyl-, monosalt,	148—149	326	325
Malonic acid, dimethyl-, monosalt,	159—160	298	294
Malonic acid, *iso*amyl-, disalt,	136—137	253	256
Malonic acid, *iso*butyl-, disalt,	136—137	246	250
Malonic acid, *iso*propyl-, disalt,	133—134	239	240
Malonic acid, *iso*propyl-, monosalt,	147—148	312	309
Malonic acid, methyl-, monosalt,	145—146	284	281
Malonic acid, methylphenyl-, monosalt,	137—138	360	364
Malonic acid, 3-nitrobenzylidene-, disalt,	146—147	285	288
Malonic acid, phenyl-, disalt,	144—145	256	258
Mandelic acid, 4-methyl-	164—165	332	328
1-Naphthoic acid,	147—148	338	341
1-Naphthoic acid, 2-hydroxy-	203—204	354	354 (D)
Nicotinic acid,	156—157	145	144
2,3-Nonenylic acid,	169—170	322	322

Table 1. continued.

Acid	m. p.	equiv. weight.	
		calc.	found
Penta-2,4-dienic acid, 2-(4-nitrophenyl)-	136—137	385	389
Phenoxyacetic acid	180—181	318	317 (E)
Phenoxyacetic acid, 2-chloro-	167—168	353	351 (F)
Phenoxyacetic acid, 4-chloro-	198—199	353	351 (G)
Phenoxyacetic acid, 2,4-dibromo-	173—175	476	474
Phenoxyacetic acid, 2,4-dichloro-	177—178	387	384
Phenoxyacetic acid, 2,4-dichloro-5-nitro-	166—167	432	429
Phenoxyacetic acid, 2-methyl-	162—163	332	329
Phenoxyacetic acid, 3-methyl-	166—168	332	329
Phenoxyacetic acid, 2-methyl-4-chloro-	164—165	367	368
Phenoxyacetic acid, 3-methyl-4-chloro-	168—169	367	363
Phenoxyacetic acid, 2-nitro-	155—156	363	359
Phenoxyacetic acid, 2,4,6-trichloro-	198—199	422	420
Phenylacetic acid, 3-methoxy-	160—161	332	333
Phenylacetic acid, 4-nitro-	166—168	347	348
Phthalic acid, 4-iodo-, monosalt,	191	457	461
Phthalic acid, mono-*n*-butylester-	137—138	388	385
Phthalic acid, mono*cyclo*hexylester-	159—160	415	412
Phthalic acid, monomethylester-	133—134	346	345
Phthalic acid, 3-nitro-, disalt,	177—178	272	273
Phthalic acid, 4-nitro-, disalt,	176—177	272	272
Phthalic acid, 4-nitro-, monosalt-	183—185	377	381
α-Picolinic acid,	185—186	14.5 %	14.4% (A)
Picric acid,	186—187	395	392
Pimelic acid, disalt,	162—163	246	245
Piperonylic acid,	172—173	332	330
Pivalic acid,	153—154	268	271
Propiolic acid, 3-phenyl-	184—186	312	308
Propionic acid, 3-benzoyl-	147—148	344	348
Propionic acid, 2-chloro-	168—169	275	278
Propionic acid, 3-chloro-	148—149	275	275
Propionic acid, 3-(4-chlorobenzoyl)-	159—160	379	374
Propionic acid, 3-*cyclo*hexyl-	175—177	323	319
Propionic acid, 2,3-dichloro-	138—139	309	305
Propionic acid, 2-ethylxantogen-	134—135	361	364
Propionic acid, 2-methylxantogen-	142—143	347	347
Propionic acid, 2-phenoxy-	154—156	332	335
Propionic acid, 3-phenyl-	151—152	316	312
Propionic acid, 2-phenyl-3-benzoyl-	158—159	421	427
Propionic acid, 3,3,3-triphenyl-	157	469	469
Pyruvic acid,	157—158	254	251
Sorbic acid,	183—184	278	279
Suberic acid, disalt,	150—151	253	252
Succinic acid, 2-chloro-, disalt,	138—139	243	243
Succinic acid, 2-methyl-, disalt,	127—129	232	234
Succinic acid, α(α-methylbenzylidene)-monoethylester-	159—160	409	412
Succinic acid, monobenzylester-	134—135	375	376
Succinic acid, monomethylester-	135—136	298	295
Succinic acid, 2-phenyl-, disalt,	164—165	263	261
cis-Δ^4-Tetrahydrophthalic acid, monosalt,	158—159	336	335
Tiglic acid,	163—164	266	263

Table 1. Continued.

Acid	m. p.	equiv. weight	
		calc.	found
2-Toluic acid, α-carboxy-, disalt,	155—156	256	253
Valeric acid, 2-chloro-	162—163	303	300
Vanillic acid,	166	334	333
Xanthic acid, methyl-	81— 83	274	277
Xanthic acid, *n*-propyl-	106—107	303	308

A) analyzed according to Dumas.
B) Donleavy [3] gives m. p. 149.
C) Tinker [5] gives m. p. 155—156.
D) Veibel [6] gives m. p. 216—217.
E) Levey and Lewis [4] m. p. 170.
F) Levey and Lewis [4] m. p. 159—159.5.
G) Levey and Lewis [4] m. p. 183.5—184.
H) Donleavy [3] gives m. p. 155.

water. The mixture is immediately cooled in ice, whereby the salt separates, often as an oil which crystallises on standing or scratching. The salts were recrystallised from 96 % ethanol, if necessary mixed with water or ether.

In case of dibasic acids it was tried to prepare both the normal and the acidic salts; the normal salts by dissolving 20 m.equiv. of the acid in 20 m.equiv. of NaOH and adding 20 m.equiv. of benzylthiuronium chloride, the acidic salts by dissolving 20 m.equiv. of the acid in 10 m.equiv. of NaOH and adding 10 m.equiv. of benzylthiuronium chloride. In most cases both procedures gave the same salt, either the normal or the acid one; only in case of *iso*propylmalonic acid, 4-nitrophthalic acid and benzoic acid, 3-sulfonic acid, could both salts be isolated. As mentioned by Berger [2], adipic acid gives both a di- and a monosalt by nearly the same procedures.

Table 2.

Acid	m. p.	equiv. weight or % nitrogen	
		calc.	found
Azobenzene, 4-dimethylamino-4′-sulfonic acid-	213—214	14.9	14.8
Azobenzene, 3,4-disulfonic acid, monosalt,	202—203	13.4	13.6
Azobenzene, 4-hydroxy-4′-sulfonic acid-,	228—229	12.6	12.6
Benzenesulfinic acid, 4-acetamino-	193—194	11.5	11.5
Benzenesulfonic acid, 4-acetamino-	248—249	11.0	11.1
Benzoic acid, 2-hydroxy-5-sulfonic acid-, disalt,	160—161	551	547 (A)
Benzoic acid, 3-sulfonic acid, disalt,	130—133	535	539 (B)
Ethylsulphuric acid,	112—113	9.6	9.3 (C)
2-Naphtol-1-sulfonic acid,	131—132	7.2	7.3
Phenol, 2-amino-4-sulfonic acid-	80— 81	11.8	11.6
Quinoline, 8-sulfonic acid-	>250	376	372
Toluene, 2-chloro-5-amino-4-sulfonic acid-,	210—215	10.8	10.6
Toluene, 4-nitro-2-sulfonic acid-,	173—174	11.0	11.0

A) Veibel [6] isolated a monosalt with m. p. 203—204.
B) Veibel [6] isolated a monosalt with m. p. 163—164.
C) Bair and Suter [1] could not prepare this derivative.

The carboxylic acid derivatives were titrated with perchloric acid in glacial acetic acid according to Berger [2], using crystal violet as an indicator. The sulfonic acid derivatives, which can not be titrated [2], were analysed (Dumas).

Derivatives of acids with strongly basic groups will titrate sharply, using two equivalents of perchloric acid; examples of this type of compounds are *m*-aminobenzoic acid, nicotinic acid and quinoline-8-sulfonic acid (the latter using one equiv. of $HClO_4$, only).

Where derivatives of acids containing less basic nitrogen are concerned, complications may arise as the basicity of the nitrogen may be high enough to allow reaction with perchloric acid, but not high enough to take up a whole equiv., resulting in an unsharp endpoint. Examples of this type of compounds, which had to be analysed (Dumas), are *o*- and *p*-aminobenzoic acid, aminochlorobenzoic acid, picolinic acid and several others.

Very weakly basic groups such as N-substituted amides, azo-groups and cyano-groups do not interfere at all in the titration.

(Microanalyses by Mr. W. Egger.)

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Kinetics of the Enzymatic Splitting of Hyaluronic Acid

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The enzymatic splitting of hyaluronic acid is studied by means of viscosimetry. The reaction is followed to more than 90 % reduction in specific viscosity. It is shown that the experiments conform in that interval with either of the following two expressions:

$$E\ t = \mathrm{A}\ (1/y - 1) + \mathrm{B}\ (1/y^2 - 1) \quad (1)$$

$$E\ t = \mathrm{C}\ (e^{\mathrm{D}y} - e^{\mathrm{D}}) \quad (2)$$

where y is a measure of the remaining viscosity and E is the total enzyme concentration. A possible reaction mechanism corresponding to the first of these expressions is discussed and some other possible mechanisms are mentioned.

Since 1934, when hyaluronic acid was first isolated by K. Meyer [1], many investigations have provided evidence of the extensive occurrence of this substance, and essential contributions have been made towards elucidation of its constitution [2]. Hyaluronidase, too, has received the attention of a great many investigators, but apart from clinical uses, the enzyme has been employed mostly as a convenient means of examining whether or not a particular liquid of high viscosity contained hyaluronic acid [3]. Much fewer investigators have been engaged in studies of the kinetic problems connected with the enzymatic decomposition of hyaluronic acid, and most of these have dealt only with splitting to the extent of 20—30 %, as a rule with the purpose of finding a convenient, fairly unique, method of standardizing a particular preparation of enzyme.

Most of the available studies of the kinetics of the reaction describe the inception of the reaction as a first-order reaction [4]. In a more recent publication, Dorfman [5] describes the reaction as a specific instance of the classical expression of Michaelis and Menten [6].

The investigations herein recorded lead to a mathematical expression by which it is possible, at the given experimental conditions, to describe more than 90 % of the reaction. This expression, as will be seen, is different from a first-order expression.

The concentration of hyaluronic acid was determined viscometrically in a modified Ostwald viscosimeter as described by Dalgaard-Mikkelsen, Kvorning and Rasbech [7]. The capacity of the apparatus was 0.6 ml; the content between the two marks was 0.2 ml. All experiments were carried out at 20° C. The flow time for distilled water was 45.0 seconds.

The substrate employed was potassium hyaluronate, produced from umbilical cords as described by Jensen [8]. The substrate as well as the enzyme were dissolved in a McIlvaine [9] phosphate-citrate buffer (0.09 M Na_2HPO_4 + 0.0105 M citric acid + 0.06 M NaCl; pH = 7.0.) The hyaluronidase used was a commercial preparation "Invasin-Lundbeck". The buffer was sterilized by heating to 100° C before use, and saturated with toluene. The viscosity of the substrate solution remained constant for several days.

Measurements were performed on a solution consisting of 0.80 % potassium hyaluronate solution and a solution of enzyme, in equal volumes. The two solutions were carefully mixed by stirring. Time was calculated to equal zero at the moment stirring was initiated. The cleansed viscosimeter was washed through with a small amount of the experimental solution and subsequently filled with 0.5 ml of it. At suitable intervals determination was made of the flow time of the solution. The time of measurement was taken as the time the solution began to run through plus half the time it occupied in running through. The viscosity at time zero was determined on a 0.40 % solution of substrate to which no enzyme was added.

The stability of the enzyme at the experimental temperature was determined in the following manner:

A solution of enzyme was placed in a thermostat at 20° C. A sample of 0.5 ml was removed and subsequently mixed with 0.5 ml of the substrate; the viscosity of the solution was then determined 3—4 times during the next fifteen minutes; the viscosimeter was cleansed and the experiment repeated at suitable intervals during the following 48 hours. As it will be seen from Fig. 1 there was no measurable alteration in the activity of the enzyme during the first 48 hours.

With a view to examining whether the chronometric integral of the reaction could be represented as $Et = f(y)$ (E = the concentration of enzyme, t = time and $1-y$ = the degree of reaction) four experiments were carried out in which the ratios of the concentrations of enzymes were 12/6/4/3. The experiments were followed until 91—94 % transformation. Fig. 2 shows viscosity as a function of time in all four experiments. In Table 1 the ratios of the periods of time consumed in obtaining a given degree of reaction are recorded for a number of different degrees of reaction. These ratios, it will be seen, were constant within about 1 %, although they had not quite the expected values. The variations can probably be accounted for by difficulty in

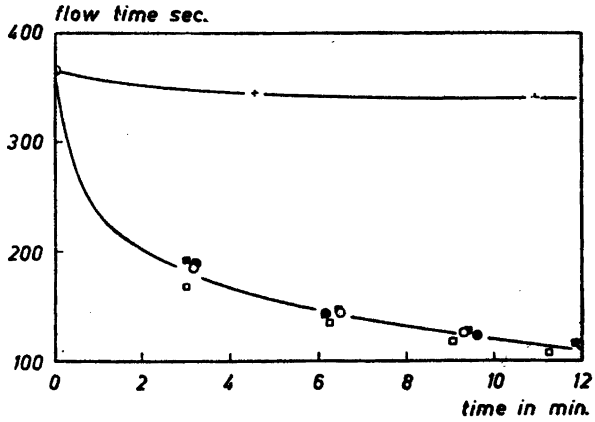

Fig. 1. Experiment with enzyme at different ages. —○—○— *6.0 min;* —□—□— *83.5 min;* —●—●— *483.0 and* —■—■— *2 872 min.* —+—+— *enzyme heated to abt. 100° C for 4 min.*

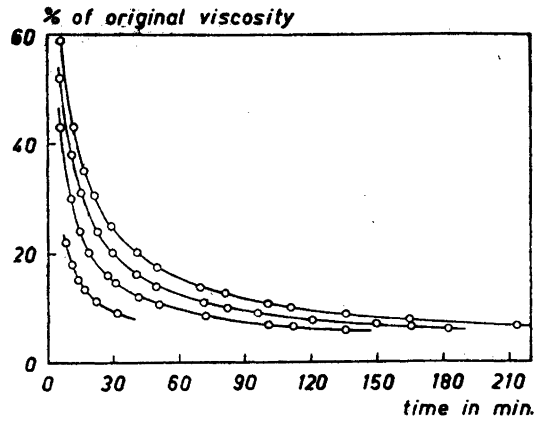

Fig. 2. Four experiments with different enzymeconcentrations.

producing solutions of enzyme exactly of the desired concentration. The constancy of the values was taken as a guarantee that the chronometric integral of the reaction could be expressed in the aforementioned general manner. The results of measurements in the experiment with enzyme concentration "4" are recorded in Table 2.

On a purely empirical basis it was found that throughout the range here examined, the reaction could be described by one of the following two expressions:

$$Et = A\ (1/y - 1) + B\ (1/y^2 - 1) \quad (1)$$

$$Et = C\ (e^{Dy} - e^{D}) \quad (2)$$

If $E = 1$ the experiment recorded in Table 2 yields the following results: A = 5.424; B = 0.339; C = 76.4 and D = 1/13.

Further, in Table 2, the differences Δ_1 and Δ_2 were found between the t-values calculated on the basis of (1) and (2), respectively, and the t-values measured.

Table 1. The table indicates the ratio between the times necessary to reach a given degree of reaction at different enzyme concentrations.

Degree of reaction	Ratio between enzyme concentrations		
	12 : 3	6 : 3	4 : 3
0.48	—	—	1.38
0.70	—	2.06	1.36
0.80	4.36	2.09	1.37
0.83	4.39	2.09	1.37
0.86	4.37	2.09	1.37
0.89	4.37	2.08	1.40
0.93	—	2.03	1.34
Average	4.37	2.07	1.37

Table 2. The table indicates (some of) the measured values for the experiment with enzyme-concentration 4. y is $\eta_{spc}/\eta^{\circ}_{spc}$, where η_{spc} is the specific viscosity of time t and η°_{spc} is the specific viscosity at zero time, and Δ_1 and Δ_2 is the difference between calculated and measured times for the mathematical expressions 1 and 2 respectively.

t min	flow time sec	y	Δ_1	Δ_2
0	555.4	1	0	0
5.89	310.9	0.5210	+0.01	+0.15
11.10	237.4	0.3770	−0.09	+0.05
15.25	204.0	0.3115	−0.11	+0.03
18.99	184.9	0.2741	−0.46	−0.35
22.74	167.2	0.2394	+0.06	+0.10
26.20	156.4	0.2183	−0.01	−0.07
29.45	148.0	0.2018	−0.02	−0.11
34.75	137.4	0.1810	−0.21	−0.45
40.36	127.6	0.1618	+0.35	+0.06
44.98	121.3	0.1495	+0.69	+0.33
49.42	116.9	0.1408	+0.44	+0.01
55.26	111.6	0.1305	+0.43	−0.02
60.49	108.6	0.1246	−0.90	−0.59
63.01	106.2	0.1199	+0.04	−0.44
70.86	101.2	0.1101	+0.60	+0.27
82.07	96.2	0.1003	−0.06	−0.09
98.08	90.5	0.0891	−0.31	+0.56
120.46	85.3	0.0789	−3.09	−0.44
133.92	82.3	0.0731	−2.15	+2.61
149.87	80.0	0.0686	−4.62	+2.09
165.5	77.5	0.0637	−2.7	+7.5
180.5	76.2	0.0611	−6.8	+6.1

Expression (1) could possibly cover a three step reaction:

$$\begin{aligned} H + x_1 &= x_2 \qquad (\pm 1) \\ H + x_2 &= x_3 \qquad (\pm 2) \\ H + x_3 &= x_1 + P \quad (\pm 3) \end{aligned}$$

where H is a molecule of hyaluronic acid, P is the reaction products and x_1, x_2 and x_3 are different combinations of enzyme and substrate. Treating this reaction as described by Christiansen [10, 11], we have, with the symbols used by Christiansen:

$$\begin{aligned} s_1 &= x_1 w_1 — x_2 w_{-1} \\ s_2 &= x_2 w_2 — x_3 w_{-2} \\ s_3 &= x_3 w_3 — x_1 w_{-3} \end{aligned}$$

and furthermore:

$$E = x_1 + x_2 + x_3$$

and in the case of stationarity:

$$s = s_1 = s_2 = s_3$$

Assuming now that w_{-3} equals zero we get by solving the equations:

$$x_1/s = 1/w_1 + w_{-1}/w_1 w_2 + w_{-1} w_{-2}/w_1 w_2 w_3 \qquad (3)$$

$$x_2/s = 1/w_2 + w_{-2}/w_2 w_3 \qquad (4)$$

$$x_3/s = 1/w_3 \qquad (5)$$

putting: $w_1 = k_1 \cdot y \cdot a$ and $w_2 = k_2 \cdot y \cdot a$ and $w_3 = k_3 \cdot y \cdot a$ where a is the initial substrate concentration and further:

$$w_{-1} = k_{-1} \text{ and } w_{-2} = k_{-2}$$

and $1/s = \mathrm{d}t/\mathrm{d}y$
we get by addition of (3), (4) and (5)

$$-E\ \mathrm{d}t/\mathrm{d}y = K_0\ 1/y + K_1\ 1/y^2 + K_2\ 1/y^3$$

where

$$K_0 = 1/k_1\ a + 1/k_2\ a + 1/k_3\ a$$
$$K_1 = k_{-1}/k_1k_2\ a^2 + k_{-2}/k_2k_3\ a^2$$
$$K_2 = k_{-1}k_{-2}/k_1k_2k_3\ a^3$$

By integration we get:

$$E\ t = K_0\ \ln\ 1/y + K_1\ (1/y - 1) + 1/2K_2\ (1/y^2 - 1)$$

Assuming that K_0 is small as compared to K_1 and K_2 we see that this expression is identical with the one found empirically when $K_1 = \mathrm{A}$ and $K_2 = 2\mathrm{B}$.

This result would be imaginable if k_{-1} is large in proportion to k_1, which would mean that the first reaction is near equilibrium.

The 3-step reaction here suggested should be taken with no slight reservation, however. Notwithstanding the fact that such a reaction may offer an explanation of the expression found, it seems rather unlikely that the reaction should, in fact, require that 3 molecules of hyaluronic acid combine with 1 molecule of enzyme before the decomposition takes place.

We have made no attempt to find a mechanism of reaction to be covered by formula (2), but we wish to draw attention to the mathematical relationship between the two expressions, as a development in series of (2) would give

$$(1-1) + (1/y-1) + K'\ (1/y^2-1) + K''\ (1/y^3-1) + \ldots$$

Neither expression (1) nor expression (2) is of the first-order form and the rectilinearity obtained by Lundquist [4] and others by plotting the logarithm of the concentration of hyaluronic acid against time cannot be claimed to support the view that we have to do with a first-order reaction. In those experiments the splitting did not exceed 30 %, and at degrees of reaction that low it is often possible to make any expression applicable to the experiment if only the right constants are chosen. In certain instances it may, of course, be convenient to use a first-order constant for the purpose of standardizing a preparation or the like, but from such experiments we cannot conclude that the reaction actually follows the pattern of a first-order reaction.

The view advanced by Dorfman [5] appears to us to be somewhat artificial, *inter alia* because it is based upon the assumption that all enzymatic processes take place in two steps, and in two steps only, which is an unpermissible abstraction from established facts. The aforementioned state of equilibrium of the first step of the reaction may be responsible for the fact that such calculations may yield satisfactory results.

One of the crucial points of all viscometric investigations of hyaluronidase is the assumption that the viscosity may readily be used instead of the concentration in the ordinary kinetic expressions. Dorfman [5], among others, has conducted experiments to show that the specific viscosity ($\eta_{\mathrm{rel}} - 1$) is directly

proportional to the concentration of hyaluronic acid. It is hardly correct, however, to assume that the viscosity measured has any relation to the actual concentration of a well defined substance. It should be taken only as a unique value which can be used to describe the reaction.

If we consider Staudinger's [12] equation $\eta_{spc} = K \cdot M \cdot c$ where M is the number of units of the high molecular substance and c the concentration in weight %, assuming then that the molecules are split near the centre [13, 14] we find that η is inversely proportional to the number of molecules, that is $\eta = K_1\ 1/n$ where n is the number of molecules and c, which is a constant for a given solution irrespective of the size of the molecules, is integrated in the constant K_1.

In the following, mention is made of a mechanism of reaction which appears to us to be more likely than the 3-step reaction, although we failed to obtain the same good agreement with the results found.

Assuming that the enzyme splits an equal number of bonds per time unit, the following expression would apply:

$$dn/dt = A \text{ (A constant)}$$

This gives $t = 1/A\ (n—n_0)$
where n_0 is the number of molecules at time zero, and n the number at time t. If n is introduced as inversely proportional to η and hence to y we get $t = B\ (1/y—1)$. It will be seen that this is the first and most important term in our expression (1). The second term of the expression, the effect of which may be said to be that more time is consumed in obtaining a given degree of reaction than would be the case if the first term were the only one, could be accounted for qualitatively in the following manner:

If the molecules of the substrate are not split at the very centre, the reduction of the viscosity, according to Staudinger's formula, would not be so great as it would be if the fragments were of equal size. The result could also be explained by imagining that the enzyme acted less on the fragments than on the larger molecules. Further, it has been established that the enzyme may also act as transglycosidase [15], and so it may also be assumed that the activity of the enzyme as such will increase with a rise in the number of fragments. One or several of the factors here mentioned might explain the occurrence of the second term of the expression.

The aspects of these possibilities are so complex, however, that we have not been able to subject them to a theoretical analysis. A promising way of attacking the problem might be to use the method described by W. Kuhn [16]. Studies of the reaction with other initial concentrations of the substrate may throw light on some of these problems.

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Studies on the Interaction of Paraffin Chain Alcohols and Association Colloids

II. The Effect of Decanol-1 on the Conductance of Fatty Acid Soap Solutions *

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The effect of decanol on the conductance of sodium oleate and sodium laurate solutions has been measured. With respect to the amount of decanol added three ranges can be distinguished in which the effect on the conductance differs. When small amounts of decanol are added that dissolve completely, the conductance decreases very little. When more decanol is added a new phase is formed and the conductance of the system decreases rapidly at first but then less rapidly. The new phase formed is composed of decanol, soap and water. Finally further additions of decanol no longer alter the conductance; in this range drops of free decanol are observed. The composition of the separated decanol-oleate-water phase has been estimated from the courses of the conductance curves. The decanol content of this phase decreases with the oleate concentration in the more dilute solutions, but remains constant at higher oleate concentrations. Below the L.A.C. no interaction occurs between decanol and association colloid and the conductance of the solution is not altered by addition of decanol. Above the C.M.C., the interaction between decanol and association colloid has a different effect on the conductance in different colloid concentration ranges; with respect to the colloid concentration, three ranges can be distinguished.

Considerable data has been accumulated on the effect of polar-nonpolar substances on the conductance of solutions of association colloids. Most of these studies have been made with compounds where the polar part of the molecule has been a carboxyl or a hydroxyl group. The great similarity of the effects of these two classes of substances has not always been taken into account.

* Part I of this series: *Acta Chem. Scand.* 7 (1953) 1098.

The earliest investigations in this field are those of McBain and co-workers [1-4] and Ekwall [5-10], who studied the effect of an excess of fatty acid on the conductance of a soap solution. Their measurements showed that the conductance of the solution first diminishes rapidly with increasing excess of fatty acid up to a certain point above which the conductance remains practically constant. At the time the first of these experiments were conducted, attention was mainly paid to the amount of added fatty acid necessary to decrease the conductance to a minimum value in order to be able to determine the composition of the acid soap formed. Ekwall [5,6] also calculated the composition of the latter from the lowering of the conductance before this minimum value was reached. He established further that the fatty acid lowered the conductance both above and below the critical micelle concentration (C.M.C.) of the soap; the interaction between fatty acid and soap was, however, found to begin only after the soap concentration had attained a certain level which was later called the limiting concentration [5,6] (or limiting association concentration, L.A.C. [11]). Below this concentration no change in the conductance was effected by the fatty acid. The value to which the conductance decreased above the L.A.C. when a sufficient amount of fatty acid was added was approximately equal to the conductance of the pure soap solution at the L.A.C. (when the original soap concentration was not much higher than the C.M.C.). Also conductometric titrations of soap solutions with hydrochloric acid conducted by Ekwall [9,10] revealed an effect of excess fatty acid on the conductance which conforms with the above results.

Recently Flockhart and Graham [12] studied the effect of a fatty acid on a soap solution (oleic acid was added to oleate solutions) in a relatively narrow concentration range below and above the C.M.C. and observed that the fatty acid effected a decrease in the conductance. Similar results were obtained in a study of the effect of propionic acid on the conductance of dodecyl sulphate solutions by Flockhart and Ubbelohde [13].

The first observations on the effect of paraffin chain alcohols on the conductance of soap solutions seem to have been reported in 1947. Ekwall [14] found that a liquid-crystalline product separated from oleate solutions when octanol or hexadecanol was added to them and that this was accompanied by a fall in the conductance. Ralston and Eggenberger [15] measured the conductances of dodecylamine hydrochloride solutions saturated with hexanol, octanol, dodecanol or octadecanol. They established that these alcohols lowered the conductance over the whole colloid concentration range studied (*i. e.* in solution both above and below the C.M.C.) and further that the C.M.C. seemed to shift simultaneously to lower concentrations. All the systems were apparently heterogeneous, milklike, viscous liquids. Brown, Grieger and Kraus [16], in a study of the influence of dodecanol on the conductance of dodecylamine hydrochloride solutions, found that the curves plotting conductance against colloid concentration for solutions containing various mole ratios of long-chain alcohol to soap had two break points. When the soap concentration was less than that corresponding to the first break point, the conductance was not altered by dodecanol. Above the first break point, the conductance decreased when dodecanol was added. When the mole ratio of dodecanol to association colloid was low (0.044 and 0.111 moles of dodecanol per mole of colloid), the mixtures seem to have been homogeneous. At higher dodecanol concentrations (mole ratio 0.24—0.26), the solutions remained clear as long as the soap concentration was high, but became turbid when the solutions were diluted. At a still higher dodecanol content (0.65 mole of dodecanol per mole of colloid), the solutions were cloudy, yet stable, over the whole range of colloid concentration studied. In these investigations a 25.6 % methanol-water mixture was used as solvent. This fact renders it difficult to compare these results with measurements made on aqueous solutions. Ekwall and co-workers [11,17,18] found that decanol does not influence the conductance of association colloid solutions when the concentration of the latter is below the L.A.C., but lowers the conductance at and above this concentration. A recently published paper by Ekwall, Passinen and Danielsson [19] deals with the effect of decanol on the conductance of oleate solutions and a paper of Heckmann [20] describes the effect of hexanol and other alcohols on the conductance of hexadecyl sulphate solutions.

Many investigations have been carried out on the effect of shorter alcohols (C_1-C_6) on the conductances of various association colloids (Brown, Grieger and Kraus [16], Bose[21-24], Flockhart and Ubbelohde [13] and others) but they are of lesser interest in connection with the present study. Neither shall we consider the studies carried out on systems containing, in addition to association colloid, water and alcohol, also a hydrocarbon or a mixture of hydrocarbons [25-27].

Also the effect of straight-chain glycols has been investigated by Ekwall and co-workers [28,18] who found that 1,10-decanediol has a negligible effect on the conductance of caprate, laurate, myristate and oleate solutions when the colloid concentration is below the L.A.C., but lowers the conductance when this concentration is exceeded; slightly above the C.M.C., however, the diol causes an appreciable increase in the conductance.

Many other types of polar-nonpolar compounds have also been observed to influence the conductance of association colloid solutions. Already in the 30's, Angelescu and coworkers [29-31] studied the effect of various cresols on the properties of soap solutions and found that the conductances of 0.1 – 0.2 M solutions first increased to a maximum and then decreased with increasing cresol content. Ekwall and Henriksson [14] followed the change in the conductance of sodium oleate solutions when terpineol was added. As long as terpineol continued to dissolve, the conductance diminished slowly, but when a new phase composed of terpineol and oleate began to separate, the conductance rapidly decreased. When still more terpineol was added, the conductance began to increase and finally attained a constant value when pure terpineol began to separate. Ralston and coworkers [32,15] found that the conductances of dodecylamine hydrochloride solutions containing methyl lauramide or lauronitrile are lower than those of solutions of the pure colloid both below and above the C.M.C. When solutions of dodecylamine hydrochloride are saturated with stearyl amine, stearonitrile or undecyl chloride, the conductance is lowered if the colloid concentration exceeds the C.M.C., but is unaffected when the colloid concentration is below this limit. McBain and McHan [33] found that dimethyl phthalate lowers the conductance of laurate solutions when the concentration of laurate is less than 0.5 M, but effects an increase when the concentration of the colloid is higher.

In this and in a following study we shall deal with the effect of the long-chain paraffin alcohols on the conductance of solutions of association colloids. In many of the above-mentioned investigations on the effect of these polar-nonpolar substances on the conductance, very little attention appears to have been paid to the physical state of the system involved, whether it has been homogeneous or not and how much of the alcohol has been added in excess of that sufficient to effect a separation of the system into two phases. These phenomena are, however, of pronounced significance for the correct interpretation of the results. Earlier studies [34,19] have shown that when the association colloid concentration is above the C.M.C., (a) relatively small amounts of decanol dissolve completely forming mixed alcohol-association colloid micelles; (b) when decanol is added in larger amounts, a new phase composed of decanol, association colloid and water separates; (c) addition of still larger amounts of decanol leads to the separation of free decanol. We have now studied the effect of decanol on the conductance of the solutions in these three ranges. We want also to elucidate how this effect changes with the concentration of the association colloid solution. In addition, we have attempted to determine the composition of the separated phase from the observed variation of the conductance. We also wish to draw attention to the great similarity between the effects of decanol and fatty acids on the conductance of association colloid solutions.

In the present paper data will be reported on the effect of decanol on the conductance of sodium oleate and sodium laurate solutions. In the following paper corresponding data relating to solutions of sodium alkyl sulphates will be presented.

MATERIALS AND METHODS

The samples of sodium oleate and decanol-1 used in this study were those used in Part I of this series [34]. The sample of sodium laurate was prepared by neutralizing purest lauric acid (Eastman Kodak Co., Rochester) with sodium ethylate in absolute ethanol.

The water employed in the preparation of the solutions had a specific conductance of $0.6-1.0 \times 10^{-6}$ reciprocal ohms. The solutions were prepared in ampoules as described in Part I, and shaken in a thermostat at 40° C during 2—4 days. Some of the solution was withdrawn into a cylindrical conductance cell and after flushing the latter several times with the solution, the conductance was measured in the usual manner; the accuracy of the conductance values was approximately ± 0.5 %. In the case of dilute oleate solutions below 0.006 M, it was difficult to obtain reproducible values, probably owing to the adsorption of decanol on the electrodes, and it was found necessary to clean the electrodes thoroughly with warm water and ethanol before introducing a new solution. The current was turned on immediately before each measurement.

In the following, the concentrations of the association colloid solutions are given in moles of soap per 1 000 g of solution and are denoted by *M*.

SODIUM OLEATE SOLUTIONS CONTAINING DECANOL

The effect of increasing amounts of decanol. As was shown in Part I of this series, sodium oleate solutions in the 0.01—0.12 *M* range solubilize a constant amount of decanol per mole of oleate before the solutions become turbid. At higher oleate concentrations, this maximum amount of decanol dissolved increases, attains a maximum in the 0.15 *M* solution and then begins to decrease.

To illustrate how the appearance of the oleate solutions changes when decanol is added, several turbidity curves are reproduced in Fig. 1. When the above-mentioned maximum amounts of decanol are exceeded and the system becomes heterogeneous, the turbidity increases very rapidly to a maximal value; when more decanol is added, the turbidity begins to decrease. The sharpness of the turbidity maximum gives the impression that a new factor begins to influence the turbidity of the solution above the maximum with the result that the turbidity decreases rapidly. The heterogeneous systems are very stable. At higher oleate concentrations they become viscous when larger amounts of decanol are introduced and gradually become gel-like. When the amount of added decanol is increased further, the turbidity again increases.

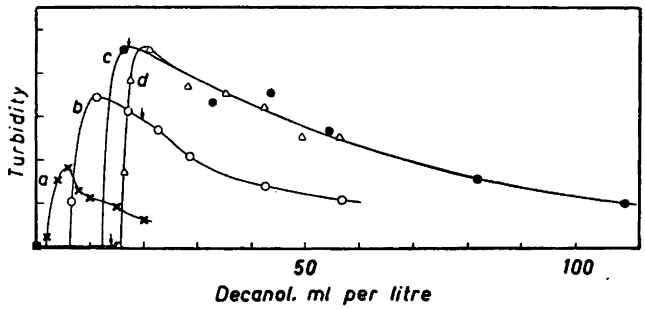

Fig. 1. Turbidity curves for sodium oleate solutions containing decanol (Pulfrich Step Photometer). 40° C.

a) 0.0500 M *sodium oleate*
b) 0.143 M » »
c) 0.275 M » »
d) 0.357 M » »

The solutions become highly viscous near the points marked with arrows.

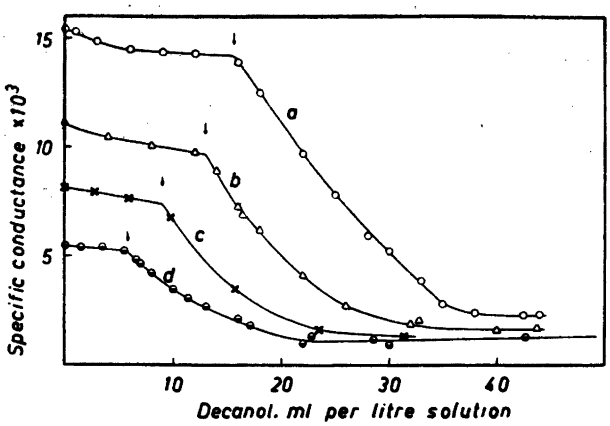

Fig. 2. Specific conductances of sodium oleate solutions containing decanol. 40° C.

a) 0.385 M *sodium oleate*
b) 0.275 M » »
c) 0.207 M » »
d) 0.143 M » »

The effect of decanol on the conductance was studied in the oleate concentration range from *ca.* 0.006 to 0.4 *M*. It could be established with certainty that decanol lowers the conductance even at the lowest oleate concentration studied (0.006 *M*).

The change of the conductance of an oleate solution varies with the amount of decanol added (Figs. 2 and 3). When small amounts are added that dissolve completely and do not give rise to turbidity, the conductance decreases gradually. When enough decanol has been added to effect the formation of a new phase (the points where turbidity is first observed are marked by arrows in Figs. 2 and 3), the conductance of the solution decreases rapidly at first but then less rapidly; finally further additions of decanol no longer alter the conductance. The gel-like nature which solutions containing large amounts of decanol often assume renders difficult the measurement of the conductance. When the oleate concentration is high, *e. g.* 0.2 *M*, this type of solution was produced already after 1 mole of decanol had been added per mole of oleate; when the oleate concentration was lower, the conductance could be measured even when the solution contained 4 moles or more decanol per mole of oleate. Observations made with the microscope revealed that drops of free alcohol were present in the systems in the range where the added decanol no longer altered the conductance.

With respect to the amount of decanol added, three different ranges can thus be distinguished in which the effect on the conductance differs. The limits of these ranges coincide with those where changes occur in the nature of the interaction between decanol and oleate.

The effect of decanol at different oleate concentrations. The curves in Figs. 2 and 3 show that as long as the added decanol does not give rise to turbidity,

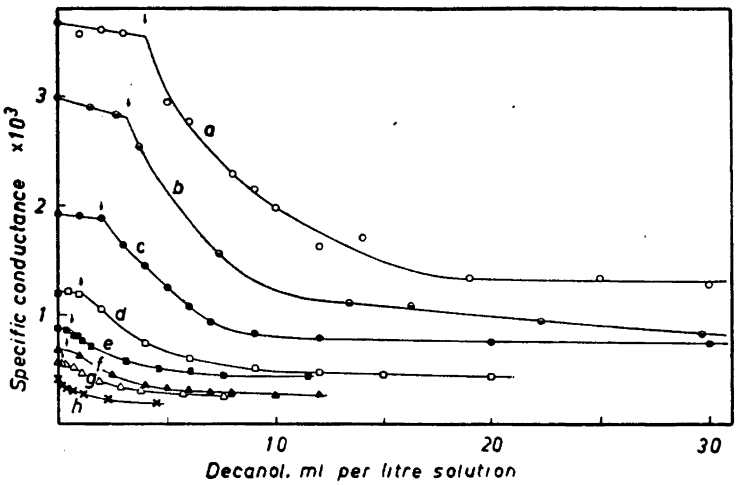

Fig. 3. Specific conductances of sodium oleate solutions containing decanol. 40° C.

a) 0.0971 M *sodium oleate*
b) 0.0782 M » »
c) 0.0500 M » »
d) 0.0300 M » »
e) 0.0199 M » »
f) 0.0150 M » »
g) 0.00998 M » »
h) 0.00592 M » »

the conductance curves have small negative slopes and are in most cases practically linear. The slope of the straight part of the curve is a measure of the effect of solubilized decanol on the conductance. From the slope we have calculated the lowering of the conductance due to solubilized decanol, when the latter is expressed in moles of decanol per mole of oleate. The slope is plotted as a function of initial oleate concentration in the upper part of Fig. 4 (curve a). Up to an oleate concentration of about 0.14 M, the absolute value of the slope is seen to increase slowly with the soap concentration; at higher oleate concentrations the slope increases more rapidly but appears to attain a maximum value in the approximately 0.3 M solution. Thus, when decanol is incorporated in the oleate micelles, it does not effect the same changes in all concentration ranges; the lowering of the conductance due to solubilized decanol is much greater for oleate concentrations above 0.2 M than for those below 0.1 M.

Curve b in the lower part of Fig. 4 gives the specific conductances of pure sodium oleate solutions (containing no decanol) and curve c the conductances of solutions containing decanol when turbidity is first observed. The difference between the conductance values of these two curves is seen to increase when the oleate concentration increases. Curve d shows on a larger scale how this difference ($\Delta\varkappa$ = the conductance of the solution containing decanol at the turbidity point *minus* the conductance of the pure solution) varies with the

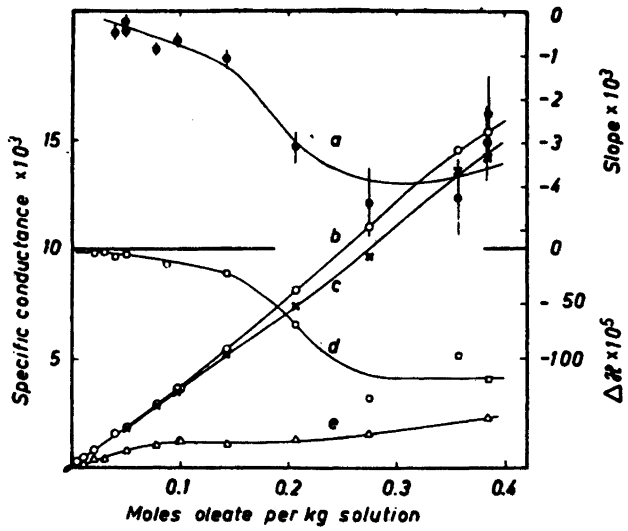

Fig. 4. a) Slopes of the conductance vs. decanol content curves below the turbidity point (Δx/mole of decanol per mole of oleate).

b) Specific conductances of pure sodium oleate solutions.

c) Specific conductances of sodium oleate solutions containing decanol at the turbidity points.

d) The lowering of the conductance, Δx (= x at turbidity point minus x of pure solution).

e) The final conductances of sodium oleate solutions containing large amounts of decanol.

oleate concentration; because this difference is negative the curve has been drawn with negative values increasing downwards. The absolute value of the difference remains relatively small until the oleate concentration rises to about 0.10—0.14 *M*, above which it increases rapidly to about six times its value in the 0.14 *M* solution and then remains relatively constant when the oleate concentration exceeds 0.27 *M*. This rapid increase of the lowering of the conductance occurs in the same oleate concentration range where the proportion of decanol in the mixed micelles increases to a maximum. As shown in Part I [34], the increase in the latter is only about 30 %, whereas we see here that the difference in the conductance increases about 500 %. It seems therefore that the change in the composition of the micelles cannot alone explain this change in the effect of decanol on the conductance.

The final values to which the conductances fall when large amounts of decanol are added are given by curve e in Fig. 4. As already seen in Figs. 2 and 3, these values are appreciably lower than those for the pure soap solutions; in magnitude they correspond to the specific conductances of 0.001—0.05 *M* oleate solutions containing no added decanol. It is of interest to note that this final conductance increases with the initial oleate concentration up to the 0.08—0.15 *M* solutions, then remains practically constant until the oleate concentration becomes approximately 0.2 *M*, after which it again increases slowly.

In respect of the oleate concentration, there thus exist three ranges in which the effect of decanol on the conductance varies in a different manner: one up to about 0.08—0.15 *M* oleate, a second from about 0.08—0.15 *M* to 0.2—0.27 *M*, and a third above the latter limit.

Estimation of the Composition of the Separated Phase. Our previous studies (Part I) have shown that interaction between decanol and oleate solutions in the small micelle range up to an oleate concentration of approximately 0.12 *M* leads primarily to the formation of mixed micelles that contain at most 0.23 mole of decanol per mole of oleate. At somewhat higher oleate concentrations, the maximum decanol content of the mixed micelles increases to about 0.30 mole of decanol per mole of oleate and then decreases to 0.14 mole of decanol per mole of oleate.

The rapid decrease of the conductance that occurs when the system becomes heterogeneous gives evidence that the phase separating contains also oleate in addition to decanol and water. By making certain assumptions, it is possible to calculate the approximate composition of the separated phase from the courses of the conductance curves. We assume that the part played by the separated phase in the transport of current can be disregarded and that the formation of this phase alters only the colloid concentration of the aqueous phase, but does not alter its micellar structure. We assume further that the volume of the aqueous phase remains unaltered. As long as the separated phase is small in amount, these assumptions may be considered valid; when larger amounts have separated, at least the last assumption is no longer true since the decanol-oleate phase that separates also contains water. The separated phase also surely participates to a certain extent in the transport of the current.

The composition of the substance causing the turbidity has been computed from the slope of the conductance curve in the region immediately above the turbidity point. The results obtained are shown by curve a in Fig. 5. It will be seen that the phase separating from the 0.1—0.4 *M* oleate solutions contains 0.5 mole decanol per mole of oleate, but that the decanol content becomes somewhat higher with decreasing oleate concentration, *e. g.* the separated phase formed from a 0.02 *M* oleate solution contains about 1.2 mole decanol per mole of oleate.

With the purpose of illustrating the calculation of the composition of the separated phase, we will take one case. From the conductance curve for the 0.0971 *M* oleate solution, it can be seen that the slope of the curve just above the turbidity point is such that an addition of 1.0 ml decanol per litre of solution decreases the conductance by $\Delta\varkappa = 0.68 \times 10^{-3}$ rec. ohms. With the aid of the conductance curve for solutions containing decanol at their turbidity points (curve c in Fig. 4), it can be estimated that this decrease in the conductance corresponds to a decrease in the oleate concentration of the aqueous phase of about 0.019 mole oleate per litre of solution. Thus an addition of 1.0 ml of decanol per litre or 0.00524 mole of decanol per litre diminishes the oleate concentration of the aqueous phase by 0.019 mole per litre. The ratio of these values is 0.28 mole of decanol per mole of oleate. To this value must be added the decanol content of the mixed micelles (0.23 mole of decanol per mole of oleate) and so we finally find that the separated phase contains 0.51 mole of decanol per mole of oleate.

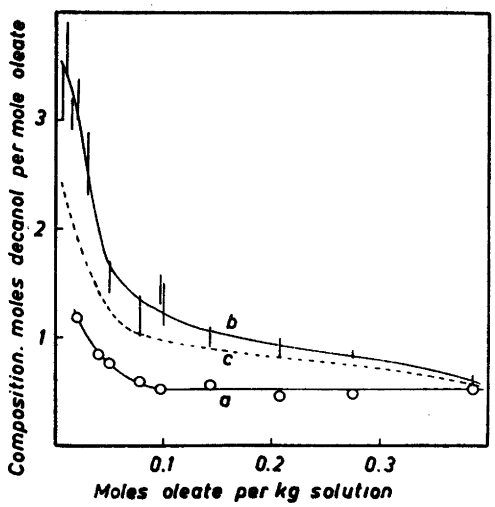

Fig. 5. The composition of the phase separating from sodium oleate solutions containing decanol.

a) At the turbidity point
b) The final composition
c) The total composition of the whole system in the range where the conductance remains constant.

Analogously, it is possible to calculate the composition of the separated phase with the aid of the latter parts of the conductance-decanol content curves. It is found that the larger the amount of decanol added to the soap solution the higher the decanol content of the separated phase. However, this increase in the proportion of decanol takes place more slowly when the concentration of oleate is high. The final composition which the separated phase tends to assume, that is, the composition when the conductance becomes constant on adding decanol, is given by curve b in Fig. 5; the conductance curves do not, however, exhibit any sharp break points, and hence it is only possible to give two limits for the final composition. Owing to the fact that the assumptions made in the calculation can no longer be considered valid (especially the assumption that the volume of the aqueous phase remains unchanged), the calculated decanol content of the separated phase is too high.

A lower limit for the final composition of the separated phase is obtained by computing the total composition of the whole system in the range where the conductance remains practically constant (curve c, dotted line, Fig. 5). The final composition of the separated phase lies somewhere between curves b and c in Fig. 5. In the most concentrated solutions, this phase hence contains 0.5—0.6 mole of decanol per mole of oleate, in the 0.05—0.1 *M* solutions, the ratio seems to be 1—1.4 moles of decanol per mole of oleate, and in the most dilute range 2—3 moles of decanol per mole of oleate.

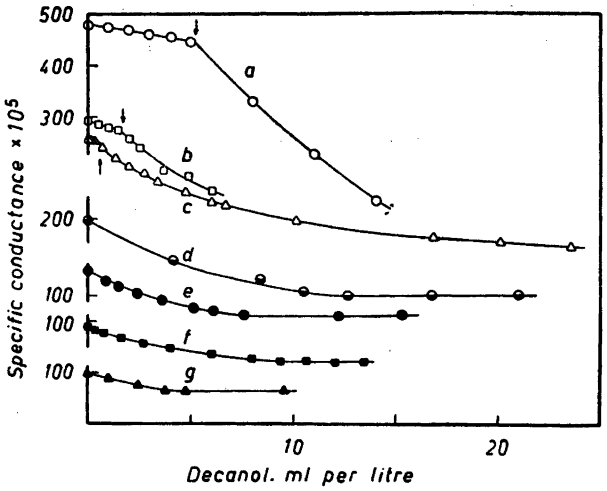

Fig. 6. Specific conductances of sodium laurate solutions containing decanol.

a)	*0.100* M	*sodium*	*laurate*	*40° C*
b)	*0.050* M	»	»	*40° C*
c)	*0.035* M	»	»	*40° C*
d)	*0.022* M	»	»	*40° C*
e)	*0.016* M	»	»	*40° C*
f)	*0.014* M	»	»	*20° C*
g)	*0.010* M	»	»	*40° C*

The present study thus shows that the phase that separates immediately above the turbidity point is appreciably richer in decanol than the mixed micelles produced by the solubilization of decanol below this point and that the decanol content of the separated phase increases further when more decanol is added to the system. Moreover it is seen that the composition of the separated phase varies with the concentration of oleate in the solution in which it is formed. Its final composition rapidly changes with oleate concentration in dilute oleate solutions, but is almost independent of the oleate concentration when the latter is high.

SODIUM LAURATE SOLUTIONS CONTAINING DECANOL

Some measurements have been made on the effect of decanol on the conductance of sodium laurate solutions in the 0.002—0.10 *M* concentration range. Small amounts of sodium hydroxide were added to most of these solutions to suppress the hydrolysis and hence the conductance values are not fully comparable.

Curves a, b and c in Fig. 6 apply to laurate solutions above the C.M.C., 0.027 *M*. Owing to the relatively high value of the C.M.C. for sodium laurate, it was possible to study the effect of decanol also in laurate solutions below the C.M.C. (curves d—g). The curves in Fig. 6 are seen to resemble those for

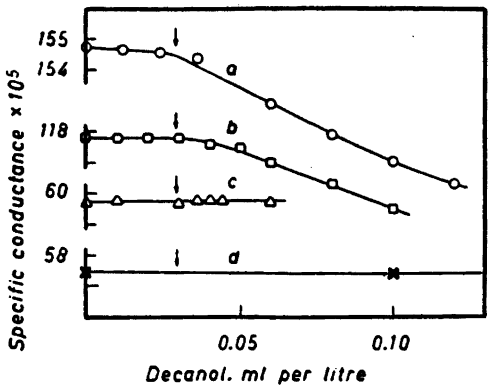

Fig. 7. Specific conductances of sodium laurate solutions containing decanol. 40° C.
a) 0.014 M *sodium laurate*
b) 0.012 M » »
c) 0.006 M » »
d) 0.005 M » »

the decanol-oleate system. As long as the solutions remain clear (the points where turbidity is observed are marked by arrows), the conductance decreases slowly with increasing decanol content, but falls rapidly when the solution becomes turbid owing to the separation of a new phase consisting of decanol, laurate and water; when greater amounts of decanol are added, the conductance approaches a constant value. Microscope observations showed that in this latter region drops of free decanol are present in the system.

A more detailed study was made both above and below the turbidity points in laurate solutions below the C.M.C. (Fig. 7). The effect of decanol is similar here as in solutions above the C.M.C., but the first part of the conductance curve is relatively short since the solution becomes turbid when very small amounts of decanol have been added (curves a and b). The lowering of the conductance is observed down to the 0.007 *M* laurate concentration. Below the limiting concentration (L.A.C., about 0.006 *M*), addition of decanol does not alter the conductance, although the system becomes heterogeneous (curves c and d, Fig. 7). In this range the separating phase is not composed of decanol, laurate and water but consists of free decanol.

We prefer to discuss the above results in the next paper of this series together with the results of similar measurements on sodium alkyl sulphate solutions.

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Studies on the Interaction of Paraffin Chain Alcohols and Association Colloids

III. The Effect of Decanol-1 on the Conductance of Sodium Alkyl Sulphate Solutions

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The effect of decanol on the conductance of sodium myristyl sulphate and sodium lauryl sulphate solutions has been measured. With respect to the amount of decanol added, three ranges can be distinguished in which the effect on the conductance differs. When small amounts of decanol are added that dissolve completely, the conductance is very little altered. When more decanol is added a new phase is formed and the conductance of the system decreases rapidly at first but then less rapidly. The new phase formed is composed of decanol, alkyl sulphate and water. Finally further additions of decanol no longer alter the conductance; in this range drops of free decanol are observed. The composition of the separated decanol-myristyl sulphate-water phase has been estimated from the courses of the conductance curves. The decanol content of this phase decreases with the myristyl sulphate concentration in the more dilute solutions, but remains constant at higher colloid concentrations. Above the C.M.C., the interaction between decanol and association colloid has a different effect on the conductance in different colloid concentration ranges; with respect to the colloid concentration, three ranges can be distinguished. These data are compared with corresponding data previously obtained for fatty acid soap solutions and are discussed in the light of other properties of association colloid solutions.

In Part II of this series [1] we described the effect of decanol on the conductance of solutions of sodium oleate and sodium laurate. We have now studied the effect of decanol on the conductance of the solutions of sodium myristyl sulphate and sodium lauryl sulphate with the purpose of determining whether this effect varies with the type of association colloid.

MATERIALS AND METHODS

The sample of sodium myristyl sulphate was the same as that used in Part I of this series [2]. The sample of sodium lauryl sulphate was prepared from Duponol P E (E. J. du Pont de Nemours & Co.) by repeated extraction with and recrystallization from absolute ethanol. The sample of *p*-xylene was a product of the Eastman Kodak Co., Rochester.

The water employed in the preparation of the solutions had a specific conductance of $0.6 - 1.0 \times 10^{-6}$ reciprocal ohms. The solutions were prepared in ampoules as described in Part I and the conductance was measured in the manner described in Part II.

In the following, the concentrations of the association colloid solutions are given in moles of colloid per 1 000 g of solution (*M*).

SODIUM MYRISTYL SULPHATE SOLUTIONS CONTAINING DECANOL

The properties of sodium myristyl sulphate solutions are modified similarly as those of oleate solutions when decanol is added to them. In myristyl sulphate solutions in the small micelle range, decanol dissolves forming clear solutions until the decanol content rises to 0.41 mole of decanol per mole of myristyl sulphate [2]. When decanol is added in excess of this ratio, the solutions become turbid (curve a, Fig. 1). The degree of turbidity increases with further addition of decanol to a maximum value (this increase in turbidity is considerably greater than in the oleate solutions) and then decreases at the same time as the solutions become more and more viscid; finally an almost transparent gel is produced, the opacity of which remains practically unchanged on further addition of decanol.

The effect of decanol on the conductance has been studied in detail only in the 0.1—0.3 *M* sodium myristyl sulphate range. In addition, conductances

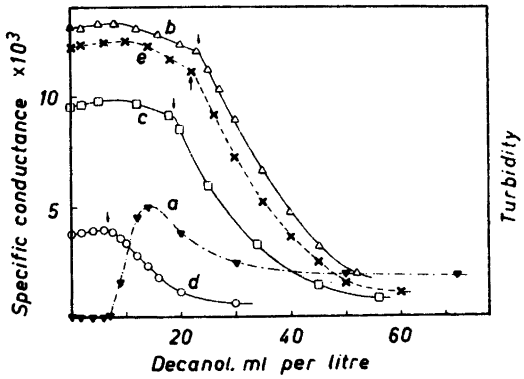

Fig. 1. a) Turbidity curve for a 0.100 M *sodium myristyl sulphate solution containing decanol. 40° C.*

Specific conductances of sodium myristyl sulphate solutions containing decanol. 40° C.
b) 0.300 M *sodium myristyl sulphate*
c) 0.232 M » » »
d) 0.100 M » » »
e) Specific conductance of 0.300 M *sodium lauryl sulphate solution containing decanol. 40° C.*

have been measured for myristyl sulphate solutions (of lower and higher concentration) in a narrow region around the point where the solutions become turbid because of the separation of a new phase on adding decanol. The conductance curves in Fig. 1 (b, c and d) show that small additions of decanol bring about small increases in the conductance of myristyl sulphate solutions, but when the myristyl sulphate concentration exceeds 0.2 *M*, further additions effect a reduction in the conductance. Above the point where the systems become heterogeneous, the variation of the conductance is fully analogous to that found for the oleate solutions. With increasing decanol content, the conductance falls rapidly at first, then more slowly until finally a point is attained where further addition of decanol alters the conductance of the solution relatively little. In this region drops of free decanol begin to separate, and if the myristyl sulphate concentration is high, the systems have a gel-like consistency.

As far as one can judge from the relatively meagre experimental data, it seems that there exists an appreciable difference between the decanol-oleate system and this system with respect to the variation of the effect of decanol on the conductance with the concentration of association colloid (Fig. 2, curves a and b). Up to a colloid concentration of about 0.2 *M*, the conductance of the myristyl sulphate solutions containing decanol at the turbidity point (curve b) is higher than the conductance of the pure solutions (curve a); only for higher colloid concentrations is the reverse true. Curve c shows on a larger scale how these changes of the conductance vary with the initial myristyl sulphate concentration. Curve d in Fig. 2 shows how the conductance varies with the myristyl sulphate concentration in the presence of large amounts

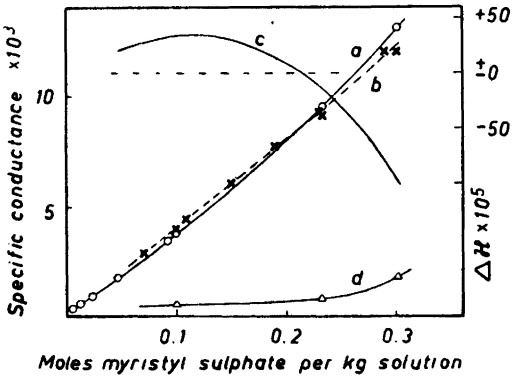

Fig. 2. a) Specific conductances of pure sodium myristyl sulphate solutions.
b) Specific conductances of myristyl sulphate solutions containing decanol at turbidity points.
c) The lowering of the conductance, Δκ (= κ at turbidity point minus κ of pure solution).
d) The final conductances of sodium myristyl sulphate solutions containing decanol.

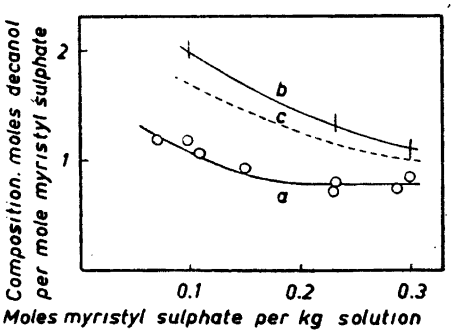

Fig. 3. The composition of the phase separating from sodium myristyl sulphate solutions containing decanol.
a) At the turbidity point.
b) The final composition.
c) The total composition of the whole system in the range where the conductance remains constant.

of decanol, *i. e.* in the range where the conductance is no longer altered by addition of decanol. This curve is similar to the corresponding curve for the decanol-oleate system.

On the basis of the available data, it appears that the composition of the separated phase varies with the myristyl sulphate concentration in a similar manner as in the decanol-oleate system (Fig. 3). In the concentration range 0.18—0.3 *M*, the phase that separates immediately above the turbidity point (curve a) contains 0.8 mole of decanol per mole of myristyl sulphate; with decreasing myristyl sulphate concentration, the ratio increases slowly to approximately 1.2 mole of decanol per moles of myristyl sulphate in the 0.07 *M* solution. The final composition attained by the separating phase when larger amounts of decanol are added (*i. e.* in the range where the conductance is no longer altered by addition of decanol) is about 1 mole of decanol per mole of myristyl sulphate in the 0.3 *M* solution and increases to about 1.5 moles per mole in the 0.2 *M* solution and to about 2 moles per mole in the 0.1 *M* solution (curve b, Fig. 3). Curve c gives the total composition of the whole system in the region where the conductance remains practically constant; it thus gives a lower limit for the final composition of the separated phase at each colloid concentration.

SODIUM LAURYL SULPHATE SOLUTIONS CONTAINING DECANOL

The effect of decanol on the conductance of a 0.3 *M* solution of sodium lauryl sulphate (curve e, Fig. 1) has also been studied. The conductance curve is similar in form to that found for the myristyl sulphate solution of the same concentration. It thus seems that a similar small increase in the conductance occurs in general within certain concentration ranges when small amounts of decanol are added to solutions of long-chain alkyl sulphates.

The maximum amount of decanol that can be incorporated in the micelles before a new phase separates is 0.38 mole per mole of lauryl sulphate (estimated from the conductance curve). Immediately above the turbidity point, the separated phase contains about 0.8 mole of decanol per mole of lauryl sulphate. The final composition of this phase seems to be about 1.0 mole of decanol per mole of lauryl sulphate.

DISCUSSION

The experimental data presented here and in Part II[1] show clearly that the effect of added alcohol on the conductance of association colloids differs greatly if the solution remains homogeneous owing to the complete solubilization of decanol in the micelles or if a new phase composed of decanol, association colloid and water or of free decanol is produced. When considering the properties of solutions containing paraffin-chain alcohols or other polar-nonpolar substances, it is hence necessary to take into account the state of the system and also the type and the degree of the interaction that takes place in the system.

Our experiments show that in the systems studied the conductance changes continuously and relatively slightly up to the point where it can be established

nephelometrically that the system becomes heterogeneous and where an abrupt decrease is noted in the conductance. Neither with the aid of a microscope with a phase contrast nor with a dark field condenser have we been able to observe any particles in the solutions below this point, whereas above it there exist not only larger particles but also a great number of very minute ultramicroscopic particles [3]. It is thus evident that below the point mentioned the system is really composed of only a single phase and that emulsified particles are not present in these solutions but only mixed micelles in thermodynamic equilibrium with the other components of the system. This is of interest as it is believed that in quaternary systems in some cases transparent emulsions are produced as a result of solubilization which are assumed to contain microscopic emulsified particles and which cannot easily be optically distinguished from solutions containing ordinary micelles [4].

As long as the system is homogeneous, the molecules of the long-chain alcohol are incorporated in the palisade layers of the micelles. If the number of the micelles remains constant, the weights and volumes of these mixed micelles must become larger than those of the original micelles; the increase in the volume is, however, to some extent counteracted by a denser packing caused by interaction between the hydroxyl groups of the alcohol molecules and the charged groups of the colloid ions [5,6]. These changes should effect a decrease in the conductance. Experiments have shown that decanol additions produce an increase in the viscosity of the solution [7,8]; this may also decrease the conductance. On the other hand, the charge density on the micelle surface becomes lower when alcohol is incorporated in the palisade layers; both this and possibly also the interaction between the hydroxyl groups and the charged groups, which may lead to the formation of hydrogen bonds, effect a decrease in the number of gegenions bound to the micelle surface. This must lead to an increase in the conductance. Probably even other factors resulting from the solubilization of the alcohol, such as changes in the form of the micelle and in the number of soap ions per micelle, can influence the conductance.

For the present, it is not possible to calculate theoretically to what extent these various factors affect the conductance. Some elementary calculations made on the basis of Stokes' formula show that the lowering of the conductance that can be ascribed to the increase in volume of the micelles due to solubilized decanol must be small if the form and the number of the micelles and the percentage of the gegenions bound to the micelle surface remain the same in colloid solutions containing decanol as in the pure colloid solutions. In the case of oleate solutions this calculated lowering of the conductance seems to be much smaller than the experimentally observed lowering.

In this connection it is of interest to compare the effects of decanol and a hydrocarbon on the conductance. Fig. 4 (curves a, b and c) shows that the conductance of sodium oleate solutions undergoes a slow decrease when *p*-xylene is solubilized. When the solutions become saturated with xylene further additions of it alter the conductance only very little (the points where saturation occurs are marked by arrows in Fig. 4). This indicates that the separating phase (xylene) contains practically no oleate. The ordinates for the curves d and e in Fig. 4 give the ratio of the conductances of oleate solutions containing xylene and decanol, respectively, to those of pure oleate solutions.

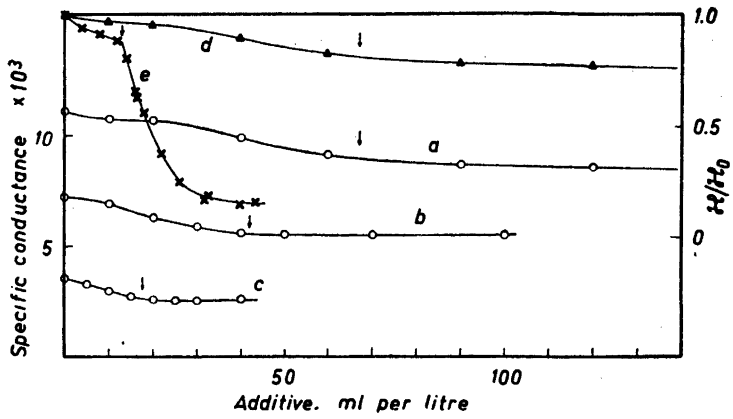

Fig. 4. Specific conductances of sodium oleate solutions containing p-*xylene. 40° C.*
a) 0.300 M *sodium oleate*
b) 0.199 M » »
c) 0.100 M » »
The ratio of the specific conductances of sodium oleate solutions containing additive to the specific conductance of the pure oleate solution, $\varkappa/\varkappa_0$.
d) 0.300 M *oleate + p-xylene*
e) 0.275 M *oleate + decanol.*

It is immediately seen that decanol has a greater effect on the conductance than the same volume of xylene, even in the range where the additives are solubilized in the micelles. (The same result is obtained if the amount of solubilized substance is expressed in grams per litre of solution or in moles per mole of oleate.) This seems to show that the increase in the volume of the micelles has only a small effect on the conductance. Calculations made on the basis of Stokes' formula show that as long as only small amounts of xylene are solubilized the calculated and experimental values of conductance are nearly equal, but that when the amount of xylene increases, the calculated values are considerably greater than the observed conductances. It therefore seems probable that the relatively small decrease in the conductance caused by small amounts of solubilized xylene is due to the increase in volume of the micelles, whereas the greater lowering of the conductance effected by decanol and by larger amounts of xylene suggests that changes also occur in the form and number of the micelles.

The effect of the liberation of the gegenions bound to the micelle surface must be considerable. If we suppose that all sodium ions initially bound by the micelles (about 50 %) would be liberated when decanol is solubilized, this would effect a large increase in the conductance. It is evidently this factor that dominates in alkyl sulphate solutions where small additions of decanol effect, as we have seen, a slight increase in conductance. In some cases the increase turns into a decrease before the turbidity point. This is presumably due to the fact that the decanol content of the micelles has become so high

that the effects that reduce the conductance begin to predominate. The recent observations of Heckmann [8], according to which addition of hexanol increases the conductance of sodium hexadecyl sulphate solutions, are evidently due to the same phenomenon. Further discussion of his results is rendered difficult by the fact that Heckmann gives no exact data on the hexanol content of the system when it becomes heterogeneous and on the composition of the separating phase.

The rapid decrease in conductance that we have observed in the case of fatty acid soap and alkyl sulphate solutions as soon as the systems become turbid owing to the separation of the decanol-association colloid-water phase is naturally due to the decrease in the colloid content of the aqueous solution. On the basis of the conductance values we have calculated that the greater part of the colloid in the aqueous solution is gradually transferred to the new phase. When further additions of decanol do not dissolve, the cause of the decrease in the conductance seems to disappear. Within the whole range above the turbidity point, the conductance changes continuously when decanol is added. No break point indicating a phase change in the emulsion is evident in the curve. This shows that even at fairly high decanol-colloid ratios, also in the gel-like systems, we are dealing with a two-phase system in which the aqueous solution is the continuous phase.

Of the four association colloids studied, only sodium laurate has been examined in concentrations below the C.M.C. The data confirm the earlier results [9] that decanol does not alter the conductance below the L.A.C. of the colloid; no interaction occurs between alcohol and soap in this range, and the excess alcohol separates in the free form [9, 10, 3]. Above the L.A.C., however, interaction is observed at all concentrations which leads to a decrease in the conductance. The amounts of decanol that are necessary to bring about the separation of a decanol-soap-water phase are very small between the L.A.C. and C.M.C.; in the vicinity of and above the C.M.C., this amount increases rapidly with increasing colloid concentration.

Above the C.M.C., the interaction between decanol and association colloid has a slightly different effect on the conductance in different concentration ranges. This is shown by the data given in Table 1, the main part of which applies to oleate solutions. Both with respect to the variation of the conductance of oleate solutions and to the composition of the separated phase as computed from the conductance values, two concentration ranges may be distinguished, one below 0.1—0.14 *M* and the other above the latter limit. The first range corresponds to that where according to our earlier measurements [2] the maximum content of decanol in the mixed micelles as calculated per mole of oleate remains constant; in the upper range this proportion increases, passes through a maximum, and then decreases. This latter range can be divided into two parts, a transition range between approximately 0.1 *M* and about 0.25 *M* where larger changes are noted, and a part above the latter limit where the observed changes are less pronounced. We should like to draw attention particularly to the fact that the different concentration ranges become clearly evident in the effect on the conductance due to solubilized decanol. This shows that the differences become evident already when relatively small amounts of decanol are incorporated in the micelles and may

Table 1. Concentration ranges in sodium oleate and sodium myristyl sulphate solutions.

Property	Sodium oleate solutions			Sodium myristyl sulphate solutions		
	Range 1	Range 2	Range 3	Range 1	Range 2	Range 3
Maximum decanol content of mixed micelles [2]	constant 0.01—0.12 *M*	passes through a maximum 0.12—0.20 *M*	decreases 0.20 *M* →	constant 0.01—0.18 *M*	passes through a maximum 0.18—0.25 *M*	decreases 0.25 *M* →
Slope of conductance *vs.* decanol content curve below the turbidity point	increases slowly 0.006—0.14 *M*	increases rapidly 0.14—0.22 *M*	remains relatively constant 0.22 *M* →			
$\Delta\varkappa = \varkappa$ at turbidity point *minus* $\varkappa$ of pure solution	negative, increases slowly 0.006—0.14 *M*	negative, increases rapidly 0.14—0.27 *M*	negative, remains relatively constant 0.27 *M* →	positive, → 0.22 *M*		negative, 0.22 *M* →
Final conductance	increases 0.006–0.10 *M*	approximately constant 0.10—0.20 *M*	increases 0.20 *M* →		approximately constant 0.1—0.23 *M*	increases 0.23 *M* →
Decanol content of separated phase at turbidity point	decreases → 0.1 *M*	remains constant 0.1—0.4 *M*		decreases → 0.18 *M*	remains relatively constant 0.18 *M* →	
Final decanol content of separated phase	decreases rapidly → 0.1 *M*	decreases slowly 0.1—0.4 *M*				

hence be attributed to differences in the original micelles in the different concentration ranges. The first concentration range coincides with the so-called small micelle range, where the properties of the micellar substance remain constant. In the upper range many of the properties differ from those in the first range; the new properties of these solutions occur at the so-called second critical concentration which lies in the above-mentioned transition range. Our results support the conception of the occurrence of a new step in micelle formation in the vicinity of this concentration limit.

Similar conclusions may be drawn in the case of myristyl sulphate solutions. In this case the following concentration ranges may be distinguished: the first below the approximately 0.18 *M* myristyl sulphate concentration and the second above the approximately 0.25 *M* concentration and a transition range between these two limits (about 0.18—0.25 *M*). Also in this case the first range coincides with the small micelle range and the transition range with the second critical concentration.

A comparison of the curves showing the effect of decanol on the conductance of solutions of the four association colloids studied reveals that the curves for the two fatty acid soaps, oleate and laurate, have completely similar courses (Part II, Figs. 2, 3, 6 and 7). The curves for the two alkyl sulphates, myristyl and lauryl sulphates, are also similar to each other (Fig. 1). Below the turbidity points, the curves for the fatty acid soaps are different from those for the alkyl sulphates but above the turbidity points the curves for the substances of these two classes have a very similar course.

Marked similarities are observed between the effects of decanol and fatty acids on the conductance of fatty acid soap solutions. This is especially the case when the acids are liquid and the reaction product, the acid soap, is liquid-crystalline. In very dilute soap solutions up to a certain limit, no change is effected by the addition; both the alcohol and the fatty acid are only emulsified. Above the concentration limit mentioned, the conductance decreases in both cases since interaction takes place that leads to the formation of a liquid-crystalline product containing soap, water and alcohol or fatty acid. This concentration limit is approximately the same in both cases; we have called it the limiting association concentration, L.A.C. Above the critical concentration increasing amounts of the polar-nonpolar substance dissolve in both cases in the solution with increasing soap concentration and are incorporated in the micelles until a point is reached where a liquid-crystalline phase begins to separate from the solution. When this separation begins, the conductance decreases abruptly. Addition of greater quantities of the polar-nonpolar substance lowers the conductance to a constant value. When the concentration of the soap solution is in the range between the L.A.C. and the C.M.C., the constant value is the same as that for the pure colloid solution at the L.A.C.; at higher soap concentrations the constant value attained is somewhat higher.

Also in respect of the composition of the separated liquid-crystalline phase, marked similarities are noted. The composition of the acid soaps has been found to vary from 0.5 mole to 2 moles of fatty acid per mole of soap. In the anisotropic decanol-soap phase, the decanol content varies from 0.5 mole to 2—3 moles per mole of soap. In both cases the separating phase also contains water.

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On the Reaction between Urea and Formaldehyde in Neutral and Alkaline Solutions

II. Experimental Studies of the Rates of the Equimolecular Reaction between Monomethylol Urea and Formaldehyde

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The paper includes the results of experimental studies at 20° C of the reaction between monomethylol urea and formaldehyde in comparatively concentrated solutions. The relationship between the amount of dimethylol urea produced by the reaction and the reaction time cannot be described by a simple second order reaction rate equation. However, an equation deduced with regard to the influence of dimethylol urea hydrolysis is in agreement with experimental results.

The reaction is found to be subject to an acid-base catalysis, but the hydroxyl ion contribution to this catalysis is not a simple function of the hydroxyl ion activity. The influence of the concentration of the reactants on the reaction rate constants was found to be small.

A study of the rates of the reaction between monomethylol urea and formaldehyde,

$$H_2N \cdot CO \cdot NH \cdot CH_2OH + HCHO = HOCH_2 \cdot NH \cdot CO \cdot NH \cdot CH_2OH$$

is due to Květoň and Králová [1]. In their investigations they used the hydroxylamine method for determining the amount of "free" formaldehyde of the reaction solutions. Since they found that this method, as it was described by Smythe [2], gives too low formaldehyde concentration data, the method was somewhat modified and highly standardized. The authors report an accuracy of $\pm$ 5% when the hydroxylamine method is used as they recommend. (However, the results obtained by Landqvist [3] in a critical analysis of some of the methods suitable for formaldehyde determinations in urea-formaldehyde reaction solutions, and the appearence of an initial rapid reaction in the experiments, as carried out by Květoň and Králová, gives the impression that $\pm$ 5 % might be a too optimistic figure when the modified method is more generally applied.)

According to Květoň and Králová, the accuracy is moderate if the later part of a reaction between urea and formaldehyde at a molar ratio of 1:2 is

used for an investigation of the reaction between monomethylol urea and formaldehyde. Thus, these authors also studied the reaction rates when starting from these latter compounds in 1 M solutions. In order to examine the influence of pH on the reaction rates, experiments were made at different pH in citric and boric acid buffers. (Corrections applied for the influence of the buffer substances on the results of the hydroxylamine titrations?) A minimum reaction rate was found at pH 6.5. All reaction rates were calculated by means of an equation corresponding to a second order reaction, and no regard was given to the influence of hydrolysis effects. Except for a rapid initial stage, the reactions were found to be of second order. However, only the middle part of the reaction was used for calculations, and when examining the rate constant data at long reaction times, deviations are found to be present. (Table 2 in the paper by Květoň and Králová.) Finally, an activation energy of 12.2 kcal was determined.

In the following the results of some experiments on the equimolecular reaction between monomethylol urea and formaldehyde are given. The purpose of these experiments has been to investigate the influence of pH and buffer substances on the reaction rates and the effect of the concentration of the reactants. A reaction rate equation will be deduced, with regard to the dimethylol urea hydrolysis.

The experimental technique was the same as previously described by Landqvist [3]. Monomethylol urea was prepared in accordance with Walter and Gewing [4]. The cryoscopic molecular weight of this monomethylol urea was 90 ± 2. The monomethylol urea was dissolved in cold water and the cryoscopic measurements made immediately in order to prevent hydrolysis effects. All experiments were carried out at $20 \pm 0.1\,^{\circ}$C and all chemicals were of A. R. quality, except for the formaldehyde, which was a Merck product of high purity, only containing traces* of formic acid and methyl alcohol.

The dry methylol urea was added to a solution containing formaldehyde and the buffer substances at the pH wanted. (The pH was then re-checked during the reaction, and no changes found, except for an experiment on an unbuffered solution, initial pH 6.70.) The addition of solid monomethylol urea was made in order to prevent any undesirable hydrolysis effects. The substance was brought into the solution and dissolved within 30—60 seconds; the reaction time was calculated from the midpoint of the time needed for complete dissolution. The maximum concentration used was 1 M, since the solubility of dimethylol urea is moderate.

When neglecting the hydrolysis of methylol ureas, we obtain the following reaction rate equation:

$$\frac{dx}{dt} = k_1 \cdot (C - x)^2 \qquad t = 0,\ x = 0 \tag{1}$$

i. e.

$$\frac{x}{C \cdot (C - x)} = k_1 \cdot t \tag{2}$$

* $< 2 \times 10^{-4}$ M at 380 g/l formaldehyde.

where x = concentration of dimethylol urea, C = initial concentration of the reactants, t = time of reaction and k_1 = reaction rate constant.

However, when regarding the hydrolysis of dimethylol urea, but still neglecting that of monomethylol urea, we find:

$$\frac{dx}{dt} = k_1 \cdot (C-x)^2 - k_2 \cdot x \qquad t = 0,\ x = 0 \tag{3}$$

where k_2 = the rate constant of the dimethylol urea hydrolysis.
We write

$$k_2/k_1 = \sigma, \textit{ i. e. } \text{the equilibrium constant.}$$

Since at equilibrium d x/d $t = 0$, we have:

$$\sigma = \frac{k_2}{k_1} = \lim_{t \to \infty} \frac{(C-x)^2}{x} \tag{4}$$

Under these conditions we obtain the following solution to eqn. (3)

$$k_1 \cdot t = \varphi = \frac{1}{\sqrt{4 \cdot C \cdot \sigma + \sigma^2}} \ln \frac{1-2 \cdot x/(2 \cdot C + \sigma + \sqrt{4 \cdot C \cdot \sigma + \sigma^2})}{1-2 \cdot x/(2 \cdot C + \sigma - \sqrt{4 \cdot C \cdot \sigma + \sigma^2})} \tag{5}$$

In each experiment the equilibrium constant was determined by extrapolation of the amount of "free" formaldehyde of the reaction solution to infinity reaction time.

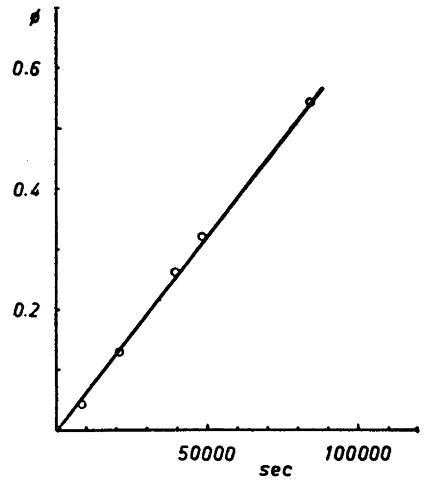

Fig. 1. Reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C in an unbuffered solution, initial pH 6.70.

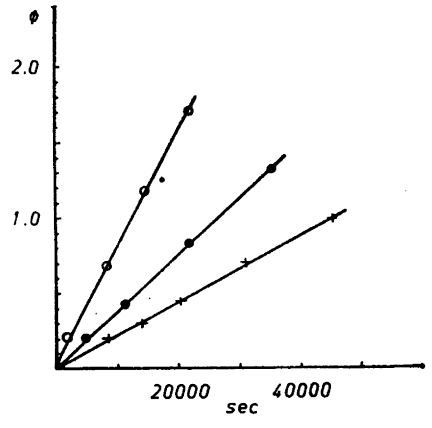

Fig. 2. Reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C and pH 6.70 at $C_{KH_2PO_4}$: 0.1 (○), 0.05 (●) and 0.025 (+).

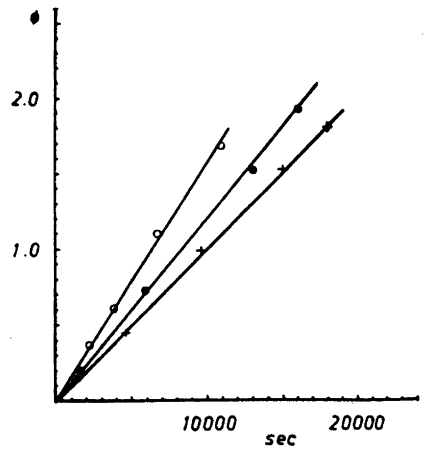

Fig. 3. Reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C and pH 9.20 at C_{borax} : 0.1 (○), 0.05 (●) and 0.025 (+).

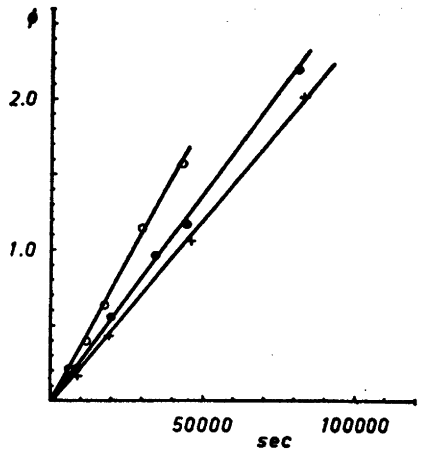

Fig. 4. Reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C and pH 10.00 at $C_{Na_2CO_3}$: 0.1 (○), 0.05 (●) and 0.025 (+).

In Figs. 1—7 the function (5) is plotted from experimental data, and in the case of Fig. 6 a comparison is also made to eqn. (2). In this investigation the reactions were continued much longer than was made by Květoň and Králová, since the later part of the reaction is of great practical interest. The composi-

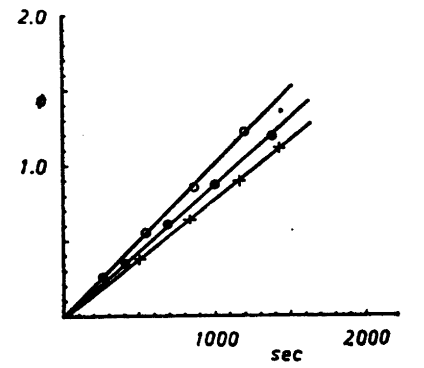

Fig. 5. Reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C and pH 11.40 at $C_{Na_2CO_3}$: 0.1 (○), 0.05 (●) and 0.025 (+).

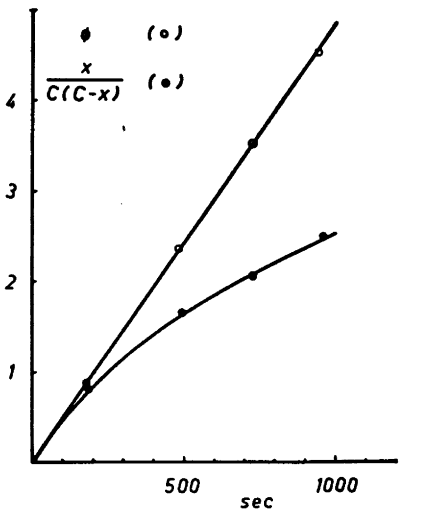

Fig. 6. Reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C and pH 12.40 and $C_{Na_3PO_4}$: 0.1.

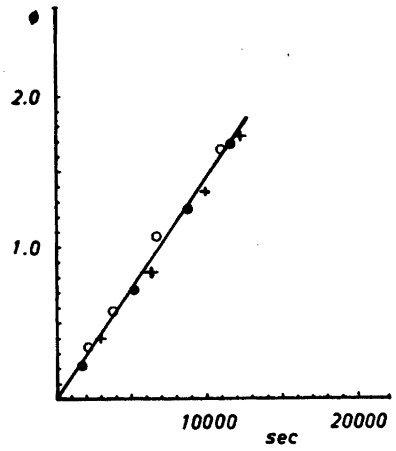

Fig. 7. Reaction at 20° C, pH 10.00 and $C_{Na_2CO_3}$ 0.1 between $H_2N \cdot CO \cdot NH \cdot CH_2OH$ and HCHO at the concentrations: 1.0 (○), 0.5 (●) and 0.25 (+).

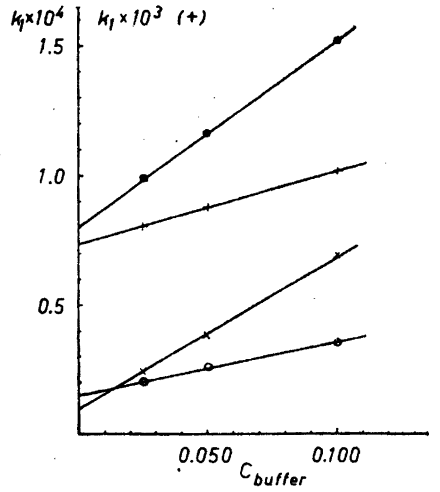

Fig. 8. Relationship between the reaction rate constant of 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C and the buffer substance concentrations: KH_2PO_4, pH 6.70 (×); borax, pH 9.20 (○); Na_2CO_3, pH 10.00 (●); Na_2CO_3, pH 11.50 (+).

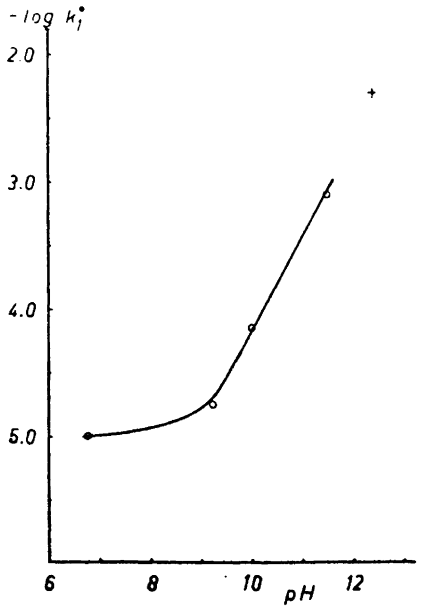

Fig. 9. Relationship between the logarithm of the reaction rate constant extrapolated to zero buffer concentration and pH for the reaction between 1.0 M $H_2N \cdot CO \cdot NH \cdot CH_2OH$ + 1.0 M HCHO at 20° C. (+) refers to 0.1 M Na_3PO_4 pH 12.40.

Table 1. Experimental data of the equimolecular reaction between monomethylol urea and formaldehyde in neutral and alkaline solutions at 20° C. In all cases σ = 0.11.

Fig.	Concentration of the reactants	Buffer substance	C_{buffer}	pH	$k_1 \times 10^4$
1	1.00	—	—	6.70 *	0.064
2	1.00	KH_2PO_4	0.100	6.70	0.79
2	1.00	KH_2PO_4	0.050	6.70	0.38
2	1.00	KH_2PO_4	0.025	6.70	0.24
3	1.00	borax	0.100	9.20	0.35
3	1.00	borax	0.050	9.20	0.26
3	1.00	borax	0.025	9.20	0.20
4	1.00	Na_2CO_3	0.100	10.00	1.52
4	1.00	Na_2CO_3	0.050	10.00	1.16
4	1.00	Na_2CO_3	0.025	10.00	0.99
5	1.00	Na_2CO_3	0.100	11.50	10.2
5	1.00	Na_2CO_3	0.050	11.50	8.7
5	1.00	Na_2CO_3	0.025	11.50	8.0
6	1.00	Na_3PO_4	0.100	12.40	48
7	1.00	Na_2CO_3	0.100	10.00	}
7	0.50	Na_2CO_3	0.100	10.00	} 1.5
7	0.25	Na_2CO_3	0.100	10.00	}

* Initial pH; final pH was 7.20.

tions of the reaction solutions and the experimental results are given in Table 1. (The cationic strength of the solutions was kept constant by adding an amount of the chloride of the buffer cation, corresponding to the difference between actual cationic strength and that of the most concentrated solution.)

When plotting the rate constants obtained at different buffer concentrations as functions of these concentrations, a relationship in accordance with Fig. 8 is found. If the curves are extrapolated to zero buffer concentration we get the sum of the "zero reaction rate" and the hydroxyl ion catalytic contribution: k_1^0. The relation between the logarithm of k_1^0 and pH is shown by Fig. 9.

From these experiments the following conclusions might be drawn, as regards the temperature and concentration range studied:

1. No linear relationship can be found when applying the simple second order reaction rate equation (Fig. 6).
2. When applying a reaction rate equation, including the regard of dimethylol urea hydrolysis, but still neglecting that of monomethylol urea, a good linearity is obtained (Figs. 1—7).
3. No rapid initial reaction can be found (Figs. 1—7).
4. The concentration of the reactants do not affect the reaction rate constants to any considerable extent (Fig. 7).

5. A linear relationship is present between the reaction rate constants and the concentration of the buffer substances at a constant cationic strength. *I. e.* the reaction is subject to an *acid-base catalysis* (Fig. 8).
6. A very low catalytic effect is present in the case of borate buffers (Fig. 3).
7. No simple relationship exists between the catalytic contribution of the hydroxyl ions and their activity (Fig. 9).

The author wishes to thank the board of *Rydboholms A. B.* for permission to publish this paper, and is also indebted to Mr. G. Johanson for his valuable assistance in the experimental work.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 1466—1470

On the Reaction between Urea and Formaldehyde in Neutral and Alkaline Solutions.

III. Experimental Studies of the Rates of Hydrolysis of Monomethylol Urea

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The rates of monomethylol urea hydrolysis at 20° C are investigated. The reaction cannot be described by a simple first order reaction rate equation, and the rate constants were determined by means of an extrapolation method applied to the first order equation relationship.

The hydrolysis is found to be subject to an acid-base catalysis, but the hydroxyl ion contribution is no simple function of the hydroxyl ion activity. The influence of the concentration of the reactants on the reaction rate constants is found to be small.

The rate of the hydrolysis of monomethylol urea:

$$H_2N-CO-NH-CH_2OH = H_2N-CO-NH_2 + HCHO$$

was studied by Crowe and Lynch [1]. The amount of liberated formaldehyde was determined by means of polarography directly in the reaction solutions. The experiment only included the hydrolysis of 0.00344 M monomethylol urea in 0.05 M LiOH; the reaction was studied by adding a monomethylol urea stock solution to an equal amount of 0.10 M LiOH. The time elapsed between the preparation of the stock solution and the addition to LiOH is not mentioned; thus it is not known if the hydrolysis occurring in the stock solution was controlled. No linear relationship was found when applying a first order reaction rate equation, but from the slope of the curve at zero reaction time, the constant was calculated.

From comparisons made between the hydrolysis rate constant obtained by this experiment and the corresponding constant of the equimolecular reaction between urea and formaldehyde an equilibrium constant of 20.5 was calculated by these authors. The equilibrium constant found from the latter of the reactions, extrapolated to infinite reaction time, was of the order 26. All data refer to 0.05 M LiOH.

The purpose of the following study is to investigate the rate of hydrolysis of monomethylol urea in comparatively concentrated solutions at 20° C with regard to the influence of pH, buffer and monomethylol urea concentration.

The experimental technique was the same as previously described (Landqvist [2]). The monomethylol urea was prepared in accordance with Walter and Gewing [3]. The cryoscopic molecular weight was found to be 90 ± 2. All hydrolysis experiments were carried out at $20 \pm 0.1°$ C, and all chemicals used were of A. R. quality. Dry monomethylol urea was added to a solution containing the buffer substances at the pH wanted and, if occurring, the very small change in pH was quickly adjusted. (The pH was re-checked during the reaction, and no changes observed, except for a reaction in an unbuffered solution with an initial pH of 6.70.) Solid monomethylol urea was added in order to prevent any influence of hydrolysis before mixing, *i. e.* in order to obtain a real zero reaction time. This time was calculated from the midpoint of the time needed for dissolving the monomethylol urea, which was of the order 30—60 seconds, since the maximum concentration of monomethylol urea was $\leqq 1.0$ M.

When neglecting the reaction between the hydrolysis products and a reaction between monomethylol urea and liberated formaldehyde producing dimethylol urea, we find the following reaction rate equation:

$$\frac{dx}{dt} = k' \, (C_0 - x) \qquad t = 0, \; x = 0 \tag{1}$$

and

$$\ln \frac{C_0}{C_0 - x} = k' \, t \tag{2}$$

where x = the concentration of urea or formaldehyde, C_0 = initial concentration of monomethylol urea, t = time of reaction and k' = the rate constant of monomethylol urea hydrolysis.

If $x \ll C_0$ we obtain:

$$\frac{x}{C_0} = k' \, t \tag{3}$$

A complete reaction rate equation requires the consideration of all the factors mentioned above. Since the rates of dimethylol urea hydrolysis are unknown, the general case cannot easily be treated. However, the rate constants can, as previously mentioned, be determined from the slope of the function (2) at zero reaction time. This method does not permit a high degree of accuracy. If the slopes of the function (2), when plotted from experimental data, were determined graphically and then plotted as functions of reaction time logarithmically, it was found that

$$\sqrt{\frac{1}{d \ln [C_0/(C_0 - x)]/dt}} = f(t) \tag{4}$$

is a suitable function for linear extrapolations to zero reaction time in the present case. By applying this method, a better accuracy could be obtained.

Table 1. Experimental data of the hydrolysis of monomethylol urea in neutral and alkaline solutions at 20° C.

$C_{H_2N.CO.NH.CH_2OH}$	Buffer substance [1]	C_{buffer}	pH	$k' \times 10^5$	$\frac{k'}{k}$ from reaction rate data
1.00	—	—	6.70 [2]	0.057	—
1.00	KH_2PO_4	0.100	6.70	0.60	0.037
1.00	KH_2PO_4	0.050	6.70	0.35	0.035
1.00	KH_2PO_4	0.025	6.70	0.22	0.034
1.00	borax	0.100	9.20	0.30	0.037
1.00	borax	0.050	9.20	0.23	0.038
1.00	borax	0.025	9.20	0.19	0.037
1.00	Na_2CO_3	0.100	10.00	0.76	0.040
1.00	Na_2CO_3	0.050	10.00	0.46	0.041
1.00	Na_2CO_3	0.025	10.00	0.35	0.035
0.50	Na_2CO_3	0.100	10.00	0.77	—
0.25	Na_2CO_3	0.100	10.00	0.77	—
1.00	Na_2CO_3	0.100	11.50	6.6	0.036
1.00	Na_2CO_3	0.050	11.50	5.8	0.036
1.00	Na_2CO_3	0.025	11.50	5.2	0.037
					Av. 0.037

[1] In all cases such amounts of a chloride of the buffer cation were added that a cationic strength corresponding to that of the most concentrated solution was obtained.
[2] Initial pH; the final pH was 7.05.

The compositions of the reaction solutions and the experimental results are given in Table 1. In this table the equilibrium constants, as calculated from the figures here obtained and the corresponding constants of the equimolecular reaction between urea and formaldehyde (Landqvist [2]), can also be found. (These data are the inverse of the figures given by Crowe and Lynch [1].) No influence of pH and the catalytic properties of the reaction solution on the equilibrium constants can be observed.

No experimental equilibrium constants have been determined, since they cannot be assumed to be similar to the constants calculated from the reaction rates. All the reactions previously mentioned might be included in the experimental equilibrium. When regarding the hydrolysis of monomethylol urea and the reaction between the liberated urea and formaldehyde, we have:

$$\frac{dF}{dt} = k' \ (C_0 - F) - k \ F^2 \qquad (5)$$

where F = the concentration of liberated formaldehyde, k = the urea-formaldehyde reaction rate constant when the reaction product is monomethylol urea. Thus, at equilibrium we obtain:

$$k'/k = F_\infty^2/(C_0 - F_\infty) \qquad (6)$$

where F_∞ = the amount of free formaldehyde at equilibrium.

During the hydrolysis the amount of free formaldehyde reaches a maximum and then decreases asymptotically; the change is very small and is probably

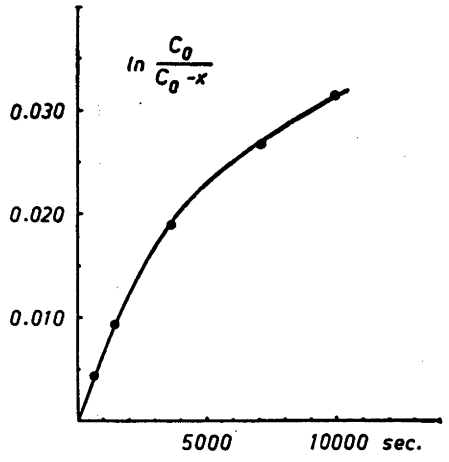

Fig. 1. The eqn. (2) relationship of the hydrolysis of 1.0 M monomethylol urea in 0.100 M Na_2CO_3, pH 10.00, 20° C.

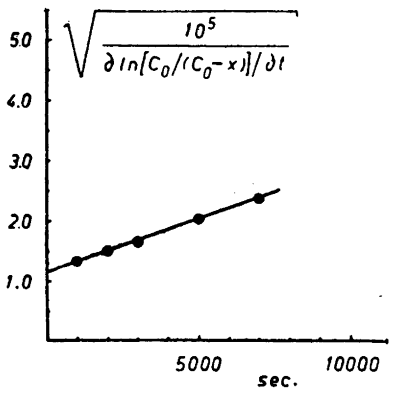

Fig. 2. The extrapolation method applied to the curve of Fig. 1.

due to dimethylol urea and urea-formaldehyde reactions. However, the amount of free formaldehyde at maximum was only about 1/4 of the amount calculated from eqn. (6) and no linear relationship was found when applying the solution to eqn. (5) to experimental data. Such results are to be expected from the previous discussion.

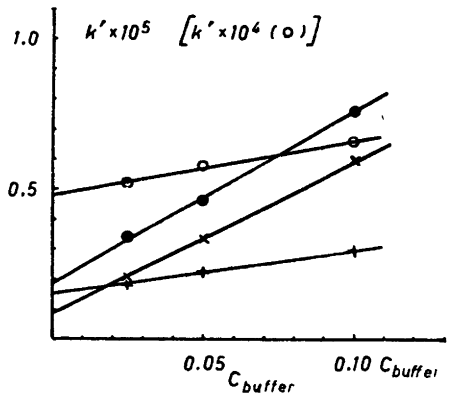

Fig. 3. Relationship between the reaction rate constants of the hydrolysis of 1.0 M monomethylol urea and the concentrations of the buffer substances at 20° C. KH_2PO_4, pH 6.70 (×); borax, pH 9.20 (+); Na_2CO_3, pH 10.00 (●) and Na_2CO_3, pH 11.50 (○).

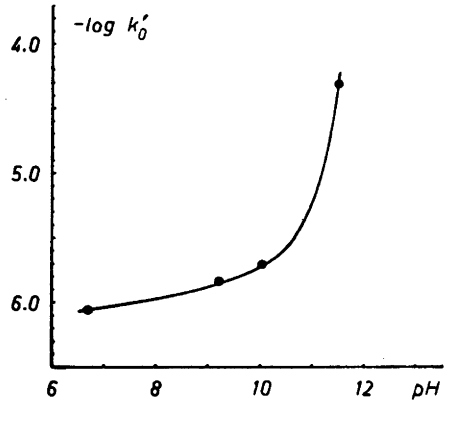

Fig. 4. Relationship between the logarithm of the reaction rate constant, extrapolated to zero buffer concentration, and pH for the hydrolysis of 1.0 M monomethylol urea at 20° C.

Fig. 1 is an example of the eqn. (2) relationships obtained, and Fig. 2 shows the corresponding extrapolation curve according to eqn. (4).

If the reaction rate constants of the monomethylol urea hydrolysis were plotted as functions of the concentrations of the buffer substances at a constant cationic strength, a relationship as shown by Fig. 3 was obtained. By extrapolation of these curves to zero buffer concentration, a "zero reaction rate", k_0', can be determined. The relation between log k_0' and pH is shown by Fig. 4.

From these experiments the following conclusions might be drawn, as regards the ranges studied:

1. No linear relationship can be found when applying a simple first order reaction rate equation (Fig. 1).
2. The equilibrium of the reaction is not in agreement with the equilibrium constants calculated from the reaction rates, and this is probably due to the influence of other reactions including mono- and dimethylol urea formation and dimethylol urea hydrolysis.
3. The concentration of the reactant does not affect the reaction rate constant to any considerable extent (Table 1).
4. A linear relationship is present between the reaction rate constants and the concentration of the buffer substances at a constant cationic strength, *i. e.* the reaction is subject to an *acid-base catalysis* (Fig. 3).
5. A very low catalytic effect is present in the case of borate buffers (Table 1).
6. The equilibrium constant, as calculated from the reaction rates, of the reaction between urea and formaldehyde producing monomethylol urea, is 0.037 (Table 1).
7. No pH dependence of the equilibrium constant seems to be present.

The author wishes to thank the board of *Rydboholms A. B.* for permission to publish this paper, and is also indebted to Mr. G. Johansson for his valuable assistance in the experimental work.

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ACTA CHEMICA SCANDINAVICA 9 (1955) 1471—1476

On the Reaction between Urea and Formaldehyde in Neutral and Alkaline Solutions

IV. Experimental Studies of the Rates of Hydrolysis of Dimethylol Urea

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The rate of hydrolysis of dimethylol urea at 20° C was studied. The reaction cannot be described by a simple first order reaction rate equation, but an equation deduced with regard to the reaction between monomethylol urea and formaldehyde is in agreement with experimental data.

The reaction is found to be subject to an acid-base catalysis, but the hydroxyl ion contribution to the catalysis is not a simple function of the hydroxyl ion activity. The influence of the concentration of the reactants on the reaction rate constants is found to be small. The equilibrium constant of the reaction between monomethylol urea and formaldehyde at 20° C is 0.11.

The rates of the hydrolysis of dimethylol urea,

$$HOCH_2 \cdot NH \cdot CO \cdot NH \cdot CH_2OH = HOCH_2 \cdot NH \cdot CO \cdot NH_2 + HCHO$$

were studied by Květŏn and Králová [1] in 0.32 M unbuffered solutions, pH 7.0, at different temperatures. The hydroxylamine titration method used by these authors for determining the amount of "free" formaldehyde of the reaction solutions was previously discussed by Landqvist [2,3], and the accuracy obtained seems to be moderate. No linear relationship was found when applying a first order reaction rate equation, but from the slopes of the curves at zero reaction time, rate constants were calculated. The activation energy was found to be 16.9 kcal.

In the following paper the results of some experimental studies of the rates of dimethylol urea hydrolysis are given. The purpose of these experiments has been to investigate the influence of pH and buffer substances on the reaction rates and the effect of the concentration of dimethylol urea. A reaction rate equation will be deduced, including the regard of the reaction between the compounds liberated during the hydrolysis.

The experimental technique was the same as previously described by Landqvist [2]. The dimethylol urea was prepared in accordance with Walter and Gewing [4]. The cryoscopic molecular weight of the product was found to be 120 ± 2. All experiments were carried out at $20 \pm 0.1°$ C, and all chemicals used were of A. R. quality.

Dry dimethylol urea was added to a solution containing the buffer substances at the pH wanted. (The pH was then re-checked during the reaction and no changes found, except for a reaction in unbuffered solution at an initial pH of 6.70.) The addition of solid dimethylol urea was made in order to get a real zero reaction time, and this time was calculated from the midpoint of the time needed for dissolving the substance, and this latter time was of the order 30—60 seconds. The moderate solubility of dimethylol urea and the need of a short dissolving time gave a maximum concentration of 0.5 M, and this figure was also the upper limit used in this investigation.

When neglecting the reaction between the hydrolysis products, we obtain the following reaction rate equation:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = k_2 \cdot (C - x) \qquad t = 0,\ x = 0 \tag{1}$$

i. e.

$$\ln \frac{x}{C - x} = k_2 \cdot t \tag{2}$$

where x = concentration of monomethylol urea or formaldehyde, C = initial concentration of dimethylol urea, t = time of reaction and k_2 = hydrolysis reaction rate constant.

When regarding the reaction between the hydrolysis products, *i. e.* the condensation reaction between monomethylol urea and formaldehyde, but still neglecting the monomethylol urea hydrolysis, we obtain:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = k_2 \cdot (C - x) - k_1 \cdot x^2 \qquad t = 0,\ x = 0 \tag{3}$$

We write $k_1/k_2 = \tau$; $\tau = 1/\sigma$, where σ is the equilibrium constant, as previously used by Landqvist [3].

Since at equilibrium $\mathrm{d}x/\mathrm{d}t = 0$, we find:

$$\tau = \lim_{t \to \infty} \frac{C - x}{x^2} \tag{4}$$

Under these conditions we get the following solution to eqn. (3)

$$k_2 \cdot t = \psi = \frac{1}{\sqrt{1 + 4 \cdot \tau \cdot C}} \ln \frac{1 + 2 \cdot \tau \cdot x/(1 + \sqrt{1 + 4 \cdot \tau \cdot C})}{1 + 2 \cdot \tau \cdot x/(1 - \sqrt{1 + 4 \cdot \tau \cdot C})} \tag{5}$$

In each experiment the equilibrium constant was determined by extrapolation to infinity reaction time.

In Figs. 1—7 the function (5) is plotted from experimental data, and Fig. 6 also includes a comparison made to eqn. (2). The compositions of the reaction

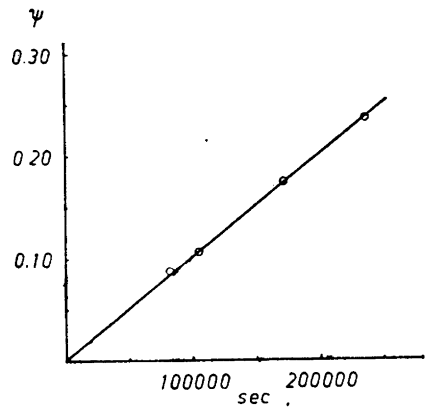

Fig. 1. Hydrolysis of 0.5 M dimethylol urea at 20° C in an unbuffered solution, initial pH 6.70.

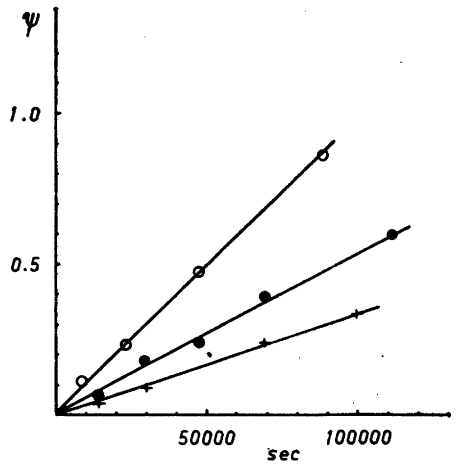

Fig. 2. Hydrolysis of 0.5 M dimethylol urea at 20° C and pH 6.70; $C_{KH_2PO_4}$: 0.1 (○), 0.05 (●) and 0.025 (+).

solutions and the experimental results are also given in Table 1. In this table the equilibrium constants, which can be calculated from the reaction rates here obtained and previously given data of the rates of the reaction between monomethylol urea and formaldehyde (Landqvist [3]), *i. e.* k_2/k_1, can also be found.

In Fig. 8 the relationship between the reaction rate constants and the buffer concentrations for each buffer substance is given. This represents the results

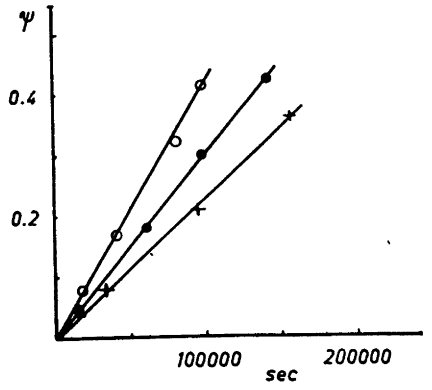

Fig. 3. Hydrolysis of 0.5 M dimethylol urea at 20° C and pH 9.20; C_{borax}: 0.1 (○), 0.05 (●) and 0.025 (+).

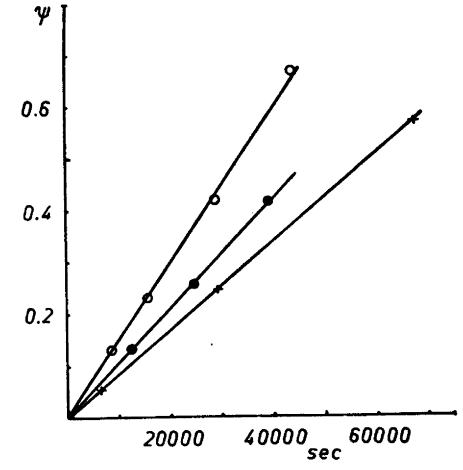

Fig. 4. Hydrolysis of 0.5 M dimethylol urea at 20° C and pH 10.00; $C_{Na_2CO_3}$: 0.1 (○), 0.05 (●) and 0.025 (+).

Table 1. Experimental data of the hydrolysis of dimethylol urea in neutral and alkaline solutions at 20° C. In all cases $\sigma = 1/\tau = 0.11$.

Fig.	Conc. of dimethyol urea	Buffer substance	C_{buffer}	pH	$k_2 \times 10^5$	σ; from reaction rate data
1	0.50	—	—	6.70 *	0.10	0.16
2	0.50	KH_2PO_4	0.100	6.70	0.98	0.12
2	0.50	KH_2PO_4	0.050	6.70	0.53	0.14
2	0.50	KH_2PO_4	0.025	6.70	0.33	0.14
2	0.50	KH_2PO_4	0.00 **	6.70	0.12	0.12
3	0.50	borax	0.100	9.20	0.42	0.12
3	0.50	borax	0.050	9.20	0.30	0.12
3	0.50	borax	0.025	9.20	0.23	0.12
3	0.50	borax	0.00 **	9.20	0.17	0.11
4	0.50	Na_2CO_3	0.100	10.00	1.45	0.11
4	0.50	Na_2CO_3	0.050	10.00	1.10	0.11
4	0.50	Na_2CO_3	0.025	10.00	0.90	0.11
4	0.50	Na_2CO_3	0.00 **	10.00	0.74	0.09
5	0.50	Na_2CO_3	0.100	11.50	11.0	0.11
5	0.50	Na_2CO_3	0.050	11.50	9.3	0.11
5	0.50	Na_2CO_3	0.025	11.50	8.3	0.10
5	0.50	Na_2CO_3	0.00 **	11.50	7.4	0.10
6	0.50	Na_3PO_4	0.100	12.40	54	0.11
6	0.50	Na_3PO_4	0.050	12.40	54	—
6	0.50	Na_3PO_4	0.025	12.40	54	—
7	0.50	Na_2CO_3	0.100	10.00		
7	0.30	Na_2CO_3	0.100	10.00	1.5	
7	0.10	Na_2CO_3	0.100	10.00		
					Average	0.12

* Initial pH; final pH was 7.50.
** Extrapolated data.

obtained at constant cationic strength, since to each solution so much of a chloride of the buffer cation was added as corresponds to the difference between actual cationic strength and that of the most concentrated solution. If the curves of Fig. 8 are extrapolated to zero buffer concentration, we get the sum of the "zero reaction rate" and the hydroxyl ion contribution: $k_2°$. The relation between the logarithm of $k_2°$ and pH is shown by Fig. 9.

As regards the equilibrium constant data, such determined by extrapolation to infinity reaction time are all 0.11. The figures calculated from reaction rate data are somewhat scattered, but the over all agreement must be regarded as good. Since the two reactions starts from two entirely different states of the reaction solutions, differences in the activity of both the reactants and the catalysts may be present.

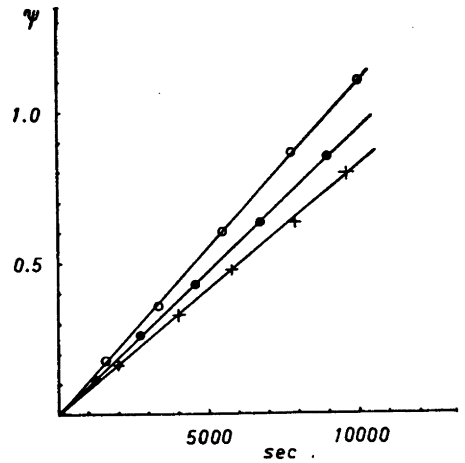

Fig. 5. Hydrolysis of 0.5 M dimethylol urea at 20° C and pH 11.50; $C_{Na_2CO_3}$: 0.1 (○), 0.05 (●) and 0.025 (+).

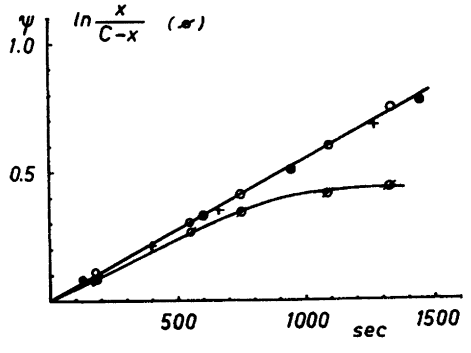

Fig. 6. Hydrolysis of 0.5 M dimethylol urea at 20° C and pH 12.40; $C_{Na_3PO_4}$: 0.1 (○), 0.05 (●) and 0.025 (+). (Φ) $C_{Na_3PO_4}$: 0.1.

From these experiments the following conclusions might be drawn, as regards the temperature and concentration range studied:

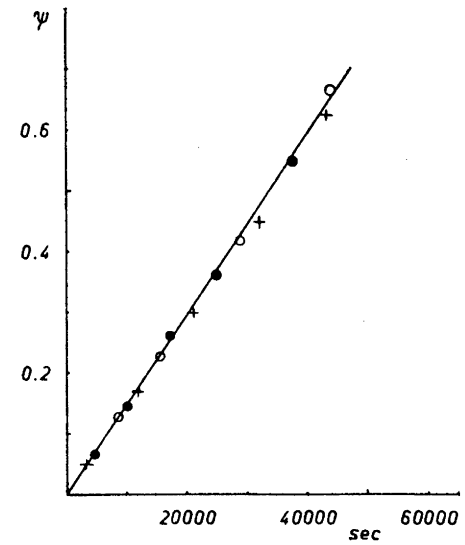

Fig. 7. Hydrolysis of dimethylol urea at the concentrations 0.5 (○), 0.3 (●) and 0.1 (+). At 20° C, pH 10.00 and $C_{Na_2CO_3}$: 0.1.

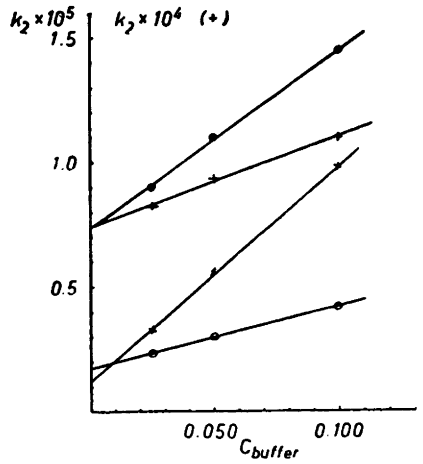

Fig. 8. Relationship between the hydrolysis rate constant of 0.5 M dimethylol urea at 20° C and the buffer substance concentrations; KH_2PO_4, pH 6.70 (×); borax, pH 9.20 (○); Na_2CO_3, pH 10.00 (●); Na_2CO_3, pH 11.50 (+).

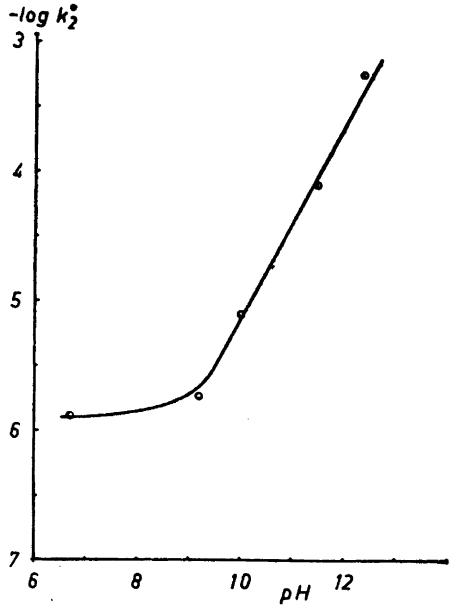

Fig. 9. Relationship between the logarithm of the reaction rate constant, extrapolated to zero buffer concentration, and pH for the hydrolysis of dimethylol urea at 20° C.

1. No linear relationship can be found when applying a simple first order reaction rate equation (Fig. 6).
2. When applying a reaction rate equation which includes the regard of the condensation reaction between monomethylol urea and formaldehyde, but still neglecting monomethylol urea hydrolysis, a good linearity is obtained (Figs. 1—7).
3. The concentration of the reactants does not affect the reaction rate constants to any considerable extent (Fig. 7).
4. A linear relationship is present between the reaction rate constants and the concentration of the buffer substances at a constant cationic strength. *I. e.* the reaction is subject to an *acid-base catalysis* (Fig. 8).
5. A very low catalytic effect is present in the case of borate buffers (Fig. 3).
6. No simple relationship exists between the catalytic contribution of the hydroxyl ions and their activity (Fig. 9).
7. The equilibrium constant of the reaction between monomethylol urea and formaldehyde is at 20° C 0.11. (Table 1 of this paper and Table 1 in Landqvist [3].) The equilibrium constant is, within the range studied, not dependent on the pH of the reaction solutions.

The author wishes to thank the board of *Rydboholms A. B.* for permission to publish this paper, and is also indebted to Mr. G. Johansson for his valuable assistance in the experimental work.

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On the Reaction between Urea and Formaldehyde in Neutral and Alkaline Solutions.

V. Experimental Studies of the Order of the "Equimolecular" Reaction between Urea and Formaldehyde at Equal Concentrations of the Reactants

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Experimental results obtained on the reaction between urea and formaldehyde at equal concentrations are analysed mathematically under the assumption that only the formation of mono- and dimethylol urea is of importance, and that the amount of dimethylol urea produced is small. A linear relationship between the reaction rate function deduced and the reaction time is present during the first 85 % of the complete reaction when previously obtained reaction rate constants are used for the calculations. From the slopes of the lines reaction rate constants are determined, and found to be in agreement with the corresponding figures obtained from reactions in solutions containing an excess of urea.

Two numerical solutions of a system of differential equations, representing the generalized reaction rate problem, are obtained by means of the modified Euler method. The "free" formaldehyde-reaction time relationship calculated from these solutions is found to be in a good agreement with the experimental results.

Finally, an empirical equation is given, suitable for practical purposes.

In a previous paper Landqvist [1] found that the equimolecular reaction at equal concentrations of urea and formaldehyde cannot be described by a second order reaction rate equation. However, if the reaction was carried out in an excess of urea, linear relationships could be obtained and reaction rate constants calculated. (The use of an excess of urea suppresses the influence of side reactions, such as dimethylol urea formation.)

In other papers by the same author the rate constants of the formation of dimethylol urea from monomethylol urea and formaldehyde [2], the hydrolysis of monomethylol urea [3] and the hydrolysis of dimethylol urea [4] were given. All reactions were found to be acid-base catalysed.

The purpose of this paper is to describe further investigations of the reaction between urea and formaldehyde at equal concentrations and to compare the experimental results with a reaction rate equation which considers the formation of mono- and dimethylol urea, but neglects the hydrolysis of these compounds; the amount of dimethylol urea produced is assumed to be small. Numerical solutions of the system of differential equations which represents the generalized reaction rate problem in this case, will be compared with the corresponding experimental results. Finally, a search for an empirical reaction rate relationship, suitable for practical purposes, will be made.

All the experiments were carried out as previously described [1].

If we only consider the formation of mono- and dimethylol urea, and assume that the amount of dimethylol urea produced is small, we get the following equation:

$$-\frac{\mathrm{d}F}{\mathrm{d}t} = k\ F^2 + k_1\ (C_0 - F)\ F \qquad t = 0,\ F = C_0 \tag{1}$$

or, when introducing $k_1/k = \beta$:

$$-\frac{\mathrm{d}F}{\mathrm{d}t} = k\ (\beta C_0\ F\ -\ (1-\beta)\ F^2) \tag{2}$$

where F = concentration of "free" formaldehyde of the reaction solution, t = time of reaction, C_0 = initial concentration of urea and formaldehyde, k = rate constant of the reaction between urea and formaldehyde producing monomethylol urea and k_1 = rate constant of the reaction between monomethylol urea and formaldehyde producing dimethylol urea.

When regarding the initial conditions, we obtain the following solution to eqn. (2):

$$\varphi = \frac{1}{\beta C_0} \ln\ (1\ -\ \beta\ +\ \frac{\beta C_0}{F}) = kt \tag{3}$$

If the experimental data are plotted in accordance with the equation of a second order reaction, and the slopes at zero reaction time are determined, these data can be used for rate constant calculations. By means of a method previously described [3], a better accuracy can be obtained. In the present case it is found that

$$\sqrt{\mathrm{d}\left[\frac{C_0 - F}{C_0 F}\right] \Big/ \mathrm{d}t} = f\ (t) \tag{4}$$

is a suitable function for linear extrapolations. By means of this the reaction rate constants were determined, and from comparisons made with the previously obtained data [2] of dimethylol urea formation, the β-values were calculated. For each buffer and pH group the average β values were used for calculations from eqn. (3), including the determination of the rate constants of the reaction producing monomethylol urea.

In Table 1 the compositions of the reaction solutions and the experimental results are given. The table also includes the data obtained from reactions in

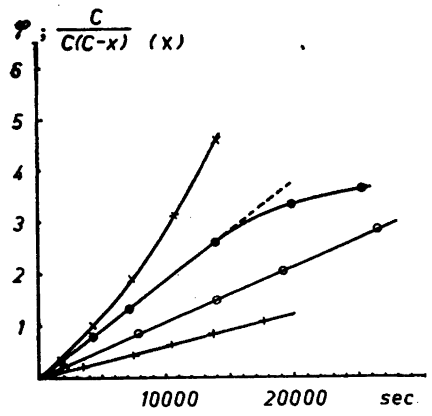

Fig. 1. The relationships in accordance with eqn. (3) of the reactions between 1 M urea and 1 M formaldehyde at 20° C, pH 6.70. $C_{KH_2PO_4}$: 0.100 (●), 0.050 (○) and 0.025 (+). The curve (×) refers to the second order reaction rate equation, 0.100 M KH_2PO_4.

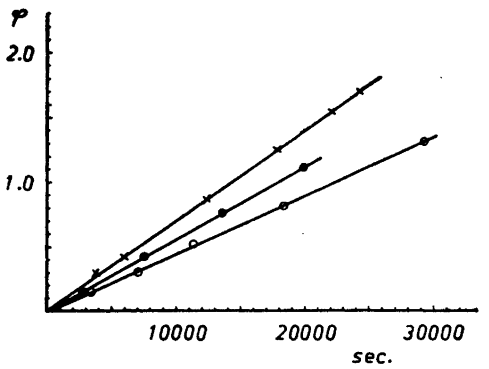

Fig. 2. The relationships in accordance with eqn. (3) of the reactions between 1 M urea and 1 M formaldehyde at 20° C, pH 9.20. C_{borax}: 0.100 (×), 0.050 (●) and 0.025 (○).

solutions containing an excess of urea, and the results are in comparatively good agreement with the present data.

In the Figs. 1—4 the plots of the φ-function are given, and it is seen that a good linearity is present during the main part of the reaction. During the last 15 % of the reactions deviations appear, which is exemplified by Fig. 1.

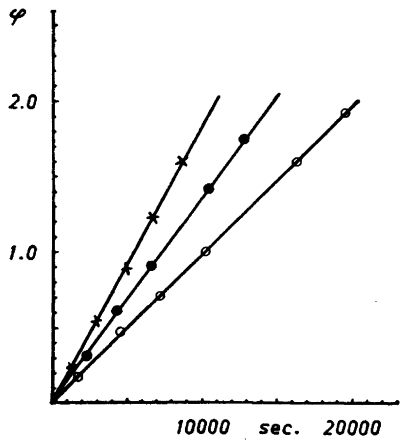

Fig. 3. The relationships in accordance with eqn. (3) of the reactions between 1 M urea and 1 M formaldehyde at 20° C, pH 10.00. $C_{Na_2CO_3}$: 0.100 (×), 0.050 (●) and 0.025 (○).

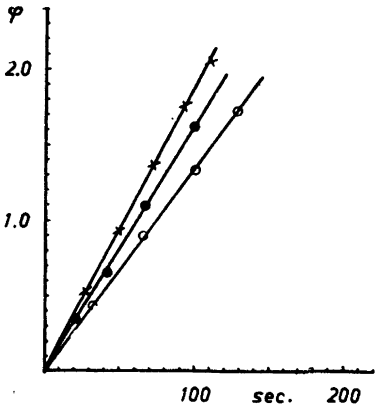

Fig. 4. The relationships in accordance with eqn. (3) of the reactions between 1 M urea and 1 M formaldehyde at 20° C, pH 11.50. $C_{Na_2CO_3}$: 0.100 (×), 0.050 (●) and 0.025 (○).

Table 1. Experimental data of the reaction between urea and formaldehyde, 20° C, at equal concentrations of the reactants.

Fig.	C_0	Buffer substance [1]	C_{buffer}	pH	β	$k \times 10^3$	$k \times 10^3$ (excess of urea)
1	1.0	KH_2PO_4	0.100	6.70	0.44	0.18	0.16
1	1.0	KH_2PO_4	0.050	6.70	0.35	0.11	0.10
1	1.0	KH_2PO_4	0.025	6.70	0.40	0.060	0.065
					0.40		
2	1.0	borax	0.100	9.20	0.50	0.070	0.080
2	1.0	borax	0.050	9.20	0.47	0.055	0.060
2	1.0	borax	0.025	9.20	0.44	0.046	0.052
					0.47		
3	1.0	Na_2CO_3	0.100	10.00	0.85	0.18	0.20
3	1.0	Na_2CO_3	0.050	10.00	0.83	0.14	0.12
3	1.0	Na_2CO_3	0.025	10.00	0.99	0.10	0.10
					0.89		
4	1.0	Na_2CO_3	0.100	11.50	0.54	19	19
4	1.0	Na_2CO_3	0.050	11.50	0.54	16	16
4	1.0	Na_2CO_3	0.025	11.50	0.54	13	13
					0.54		

[1] In all cases such amounts of a chloride of the buffer cation were added that the cationic strength corresponding to that of the most concentrated solution was obtained.

In the same figure an example is given of the results of the use of a simple second order equation, *i. e.* no linearity is obtained.

The deviations mentioned are, of course, due to the approximations introduced in eqn. (1), *i. e.* the hydrolysis of the reaction products cannot be neglected, and further, the amount of dimethylol urea produced cannot be regarded as small.

During the reaction between urea and formaldehyde at equal concentrations, the following reactions are possible:

$$H_2N-CO-NH_2 + HCHO = H_2N-CO-NH-CH_2OH \quad (A)$$
$$H_2N-CO-NH-CH_2OH + HCHO = HOCH_2-NH-CO-NH-CH_2OH \quad (B)$$
$$H_2N-CO-NH-CH_2OH = H_2N-CO-NH_2 + HCHO \quad (C)$$
$$HOCH_2-NH-CO-NH-CH_2OH = H_2N-CO-NH-CH_2OH + HCHO \quad (D)$$

When considering all of these reactions we have:

$$\left.\begin{aligned} \frac{\partial M}{\partial t} &= k\ U\ F - k_1\ M\ F - k'\ M + k_2\ D \\ \frac{\partial D}{\partial t} &= k_1\ M\ F - k_2 D \end{aligned}\right\} \quad (5)$$

Initial conditions: $t = 0$, $U = C_0$, $F = C_0$, $M = 0$ and $D = 0$.
Boundary conditions: $t \to \infty$ $\partial M/\partial t = \partial D/\partial t = 0$.

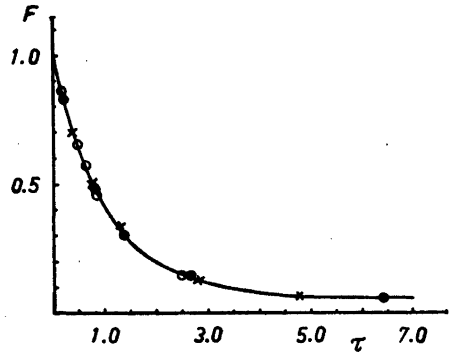

Fig. 5. The concentration of "free" formaldehyde as a function of the τ-value from a numerical solution to eqn. (6). Reaction solution: 1 M urea and 1 M formaldehyde, 20° C, pH 6.70, KH_2PO_4 buffer. Experimental data: $C_{KH_2PO_4}$: 0.100 (×), 0.050 (●) and 0.025 (○).

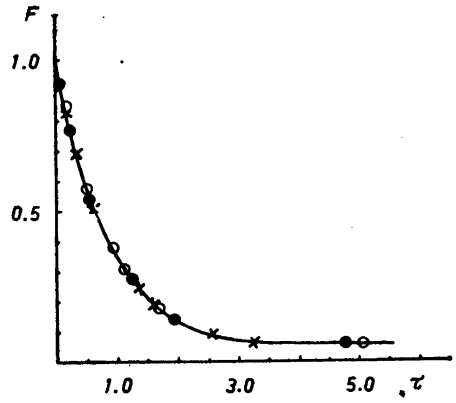

Fig. 6. The concentration of "free" formaldehyde as a function of the τ-value from a numerical solution to eqn. (6). Reaction solution: 1 M urea and 1 M formaldehyde, 20° C, pH 10.00, Na_2CO_3 buffer. Experimental data: $C_{Na_2CO_3}$: 0.100 (×), 0.050 (●) and 0.025 (○).

were C_0 = initial concentrations of urea and formaldehyde, U = concentration of urea, F = concentration of formaldehyde, M = concentration of monomethylol urea, D = concentration of dimethylol urea, t = time of reaction, k = rate constant of reaction (A), k_1 = rate constant of reaction (B), k' = rate constant of reaction (C) and k_2 = rate constant of reaction (D).

Since

$$U = C_0 - M - D \qquad \text{and} \qquad F = C_0 - M - 2D$$

and when introducing

$$k\ t = \tau$$

we get:

$$\left.\begin{aligned} \frac{\partial M}{\partial \tau} &= a + b\ M + c\ M^2 + d\ M\ D + e\ D + f\ D^2 \\ \frac{\partial D}{\partial \tau} &= g\ M + h\ M^2 + i\ M\ D + j\ D \\ F &= C_0 - M - 2D \end{aligned}\right\} \qquad (6)$$

When considering the initial conditions, we find the following constants:

$a = C_0^2$, $b = -((2 + \beta)\ C_0 + \alpha)$, $c = 1 + \beta$, $d = 3 + 2\ \beta$, $e = -(3\ C_0 - \gamma)$, $f = 2$, $g = \beta\ C_0$, $h = -\beta$, $i = -\ 2\ \beta$, and $j = -\gamma$;
where $\alpha = k'/k$, $\beta = k_1/k$ and $\gamma = k_2/k$.

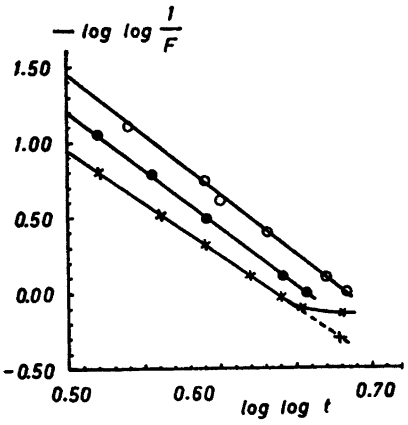

Fig. 7. The empirical expression (7) applied to the reaction between 1 M urea and 1 M formaldehyde at 20° C, pH 6.70. $C_{KH_2PO_4}$: 0.100 (×), 0.050 (●) and 0.025 (○). (+) refers to eq. (8), the equilibrium concentration of formaldehyde is taken from experimental data.

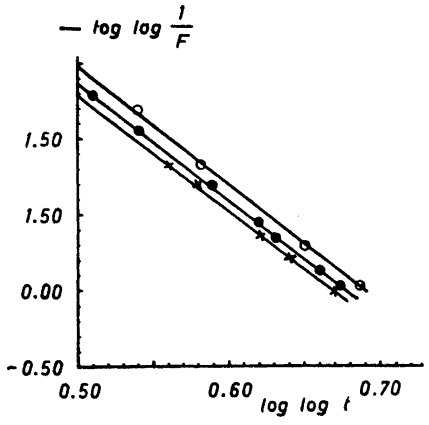

Fig. 8. The empirical expression (7) applied to the reaction between 1 M urea and 1 M formaldehyde at 20° C, pH 9.20. C_{borax}: 0.100 (×), 0.050 (●) and 0.025 (○).

Numerical solutions to this system of differential equations can be obtained by means of the modified Euler method (See *e. g.* Margenau and Murphy [5]). The *F*-reaction time relationship was calculated for the cases 1.0 M urea + 1.0 M formaldehyde in KH_2PO_4, pH 6.70 (Fig. 5) and in Na_2CO_3, pH 10.00 (Fig. 6)

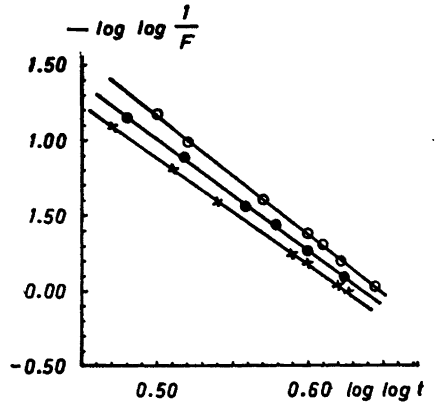

Fig. 9. The empirical expression (7) applied to the reaction between 1 M urea and 1 M formaldehyde at 20° C, pH 10.00. $C_{Na_2CO_3}$: 0.100 (×), 0.050 (●) and 0.025 (○).

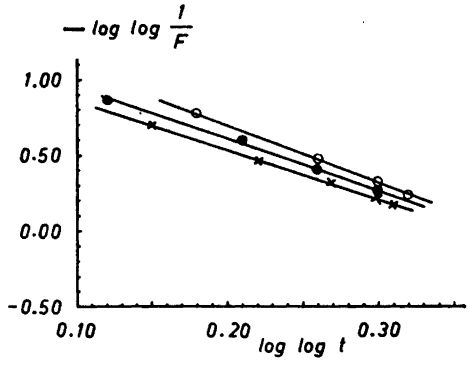

Fig. 10. The empirical expression (7) applied to the reaction between 1 M urea and 1 M formaldehyde at 20° C, pH 11.50. $C_{Na_2CO_3}$: 0.100 (×), 0.050 (●) and 0.025 (○).

using previously obtained data [1-4] of k, k_1, k' and k_2. In the diagrams 5 and 6 experimental data are also plotted, and it is seen that a very good agreement is present. Thus, the generalized solution to the rate problem describes the reactions with a high degree of accuracy. It can also be mentioned that experimentally determined formaldehyde concentrations at equilibrium are the same as obtained from 1.0 M monomethylol urea hydrolysis experiments [3].

However, for practical purposes an empirical equation which describes the amount of "free" formaldehyde of the reaction mixtures as functions of the reaction times would be of a great value. From logarithmical plots it is found that such a relationship is given by:

$$\log \log \left| \frac{F_0}{F} \right| = \mathrm{A} \log \log t + \mathrm{B} \qquad (7)$$

Here A and B are empirical constants. F_0 is the initial formaldehyde concentration.

This equation can be further refined if we introduce the equilibrium concentration of formaldehyde, F_∞, which refers to infinite reaction time. Then we have:

$$\log \log \frac{F_0}{F - F_\infty} = \mathrm{A} \log \log t + \mathrm{B} \qquad (8)$$

I Figs. 7—10 the plots of the eqn. (7) are given, and it is seen that a good linearity is obtained. In Fig. 7 an example is given, which shows that the expression (7) is also valid close to equilibrium, and that the range of validity is further extended if eqn. (8) is applied.

The practical value of an empirical expression of this kind is not only the characterization of the reactions, but also the possibility of linear extrapolations during the run of a technical reaction.

Finally, the difference between actual and eqn. (8) behaviour can be used for determination of the equilibrium formaldehyde concentrations, since the value of F_∞ necessary for a linear relationship can easily be determined.

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A Rapid Method for the Determination of Uranium

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A method is given for the determination of uranium in solutions with a low concentration of uranium and a high concentration of iron, aluminium, magnesium and sulphate. The uranium is selectively adsorbed from sulphate solutions at pH = 2 by a strongly basic anion exchanger, *e.g.* Dowex 2, in sulphate form. The influence of pH, sulphate concentration and valence state of uranium was studied.

The adsorbed uranium is eluted with dilute hydrochloric acid and determined spectrophotometrically.

The spectrophotometric method using sodium hydroxide and hydrogen peroxide for the uranium determination has been modified to give accurate results even in the presence of certain amounts of iron.

A method has been developed by which a determination of uranium in aqueous solutions containing down to 0.001 % U together with a number of other metal ions can be made in 1½—2 hours. The method has been used as a routine method in the central analytical laboratory of AB Atomenergi, Stockholm, Sweden, for the last few years with satisfactory results.

The method is based on the fact that, when a solution containing uranium sulphate passes through a column of a strongly basic anion exchanger, *e.g.* Dowex 2, the uranium is selectively adsorbed by the ion exchanger. The adsorbed uranium can be eluted by different agents and determined by a suitable method.

The spectrophotometric determination of uranium in a solution of NaOH and H_2O_2[1] proved to be satisfactory in this case. By following the procedure given below, moderate concentrations of iron, chromium, vanadium, molybdenum, manganese and phosphate do not interfere.

The degree of adsorption of hexavalent uranium on a strongly basic anion exchanger from a solution containing sulphate is dependent on the hydrogen ion concentration and the concentration of the sulphate. (Figs. 1 and 2.) A quantitative adsorption from a solution of uranyl sulphate is obtained, when the pH of the solution is higher than about 1.2. In the presence of a concentration of 200 g $NaHSO_4$/litre, a quantitative adsorption of the uranium occurs when the pH is about 2 or higher. A practical upper limit for the useful pH-

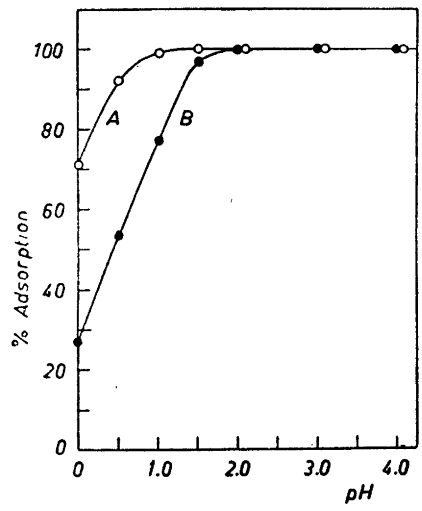

Fig. 1. The influence of pH on the adsorption of uranium. A 20 ml column of Dowex 2 in sulphate form was used. Grain size: 0.2—0.4 mm. Flow rate: 4—5 ml/min. Curve A: 248 mg uranium (VI) as sulphate in aqueous solution + H_2SO_4 for the adjustment of pH. Volume of solution: 65 ml. Curve B: 248 mg uranium (VI) as sulphate in aqueous solution + 16 g Na_2SO_4 + H_2SO_4 for the adjustment of pH. Volume of solution: 65 ml.

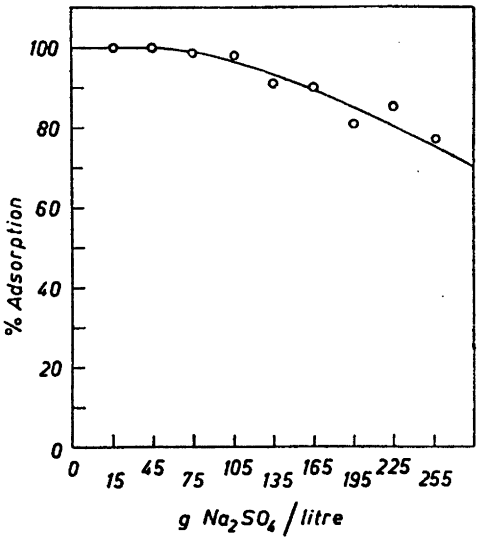

Fig. 2. The influence of the concentration of Na_2SO_4 on the adsorption of uranium (VI) at pH = 1. A 20 ml column of Dowex 2 in sulphate form was used. Grain size: 0.2—0.4 mm. Flow rate: 4—5 ml/min. Aqueous solutions of 248 mg uranium (VI) as sulphate + varying amounts of Na_2SO_4 + H_2SO_4 for the adjustment of pH. Volume of solution: 65 ml.

range is about 4, above which precipitation phenomena take place. Since those solutions, which are subject to routine determinations of uranium and for which the present method was developed, contain a concentration of sulphate equivalent to about 200 g $NaHSO_4$/l, pH equal to 2 was chosen for the preliminary experiments on the adsorption of uranium.

Uranium(IV) is not adsorbed quantitatively from a sulphate solution under those conditions which secure a quantitative adsorption of uranium (VI), (Fig. 3).

The presence of chloride ion decreases the adsorption of uranium (Table 1).

The adsorbed uranium is eluted in a suitable manner with dilute hydrochloric acid. As seen from Fig. 4 the volume required for 98 % recovery of the adsorbed uranium is about the same for concentrations of 1 : 20, 1 : 10 and 1 : 5. When hydrochloric acid 1 : 1 was used, 75 ml eluted about 5 % of the adsorbed uranium. Further addition of the latter elutriant effected practically no elution. The residual uranium was retained by the ion exchanger as a chloride complex.

The adsorption of chromium and vanadium, which both severely interfere with the spectrophotometric determination of uranium with NaOH and H_2O_2,

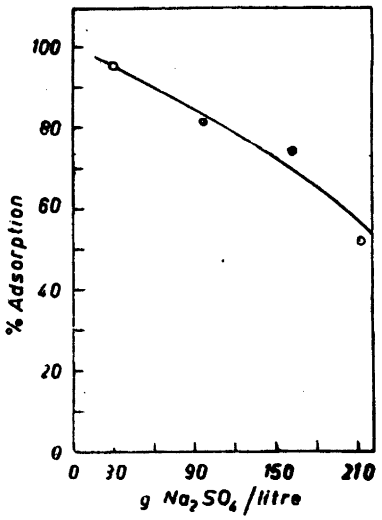

Fig. 3. The influence of the concentration of Na_2SO_4 on the adsorption of U (IV) at pH = 2. A 5 ml column of Dowex 2 was used. Grain size: 0.2—0.4 mm. Flow rate: 4—5 ml/min. Aqueous solutions of 9.6 mg U(IV) as sulphate + varying amounts of Na_2SO_4 + H_2SO_4 for the adjustment of pH. Volume of solution: 60 ml.

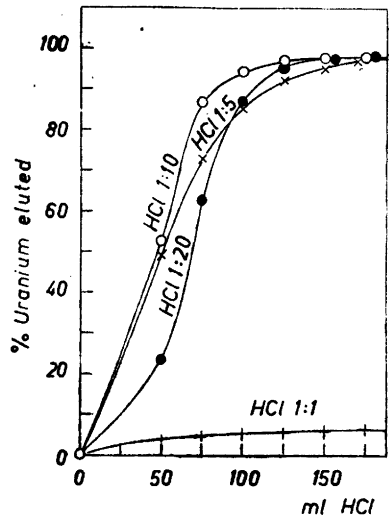

Fig. 4. The influence of the concentration of HCl on the elution of 248 mg uranium adsorbed on 20 ml Dowex 2, 0.2—0.4 mm, in sulphate form. Flow rate: 4—5 ml/min.

was found to be nil at pH = 2, provided that chromium was present as chromium(III) and vanadium as vanadium(IV). Iron(II) is not adsorbed but iron(III) is partially retained by the ion exchanger. More than 90 % of the trivalent iron passes through the ion exchanger without being adsorbed, when

Table 1. The adsorption of uranium(VI) as sulphate by 20 ml Dowex 2, 0.2—0.4 mm, in sulphate form. Flow rate: 4—5 ml/min. The solutions had a volume of 65 ml, a pH = 2 and contained 248 mg U(VI) and also Na_2SO_4 and NaCl.

g Na_2SO_4	g NaCl	% Uranium adsorbed
15.5	0.4	99.5
15	0.8	98.2
14.5	1.2	96.2
14	1.6	82.0
13	2.5	69.3
12	3.5	58.6
10	4.9	31.2
8	6.6	27.1
6	8.2	20.0
4	9.9	13.0
2	11.5	24.9
0	13.2	26.5

Table 2. The adsorption of iron(III) from sulphate solutions.

mg Fe^{3+} added	g Na_2SO_4 added	Iron adsorbed mg	Iron adsorbed %
100	—	22	22
200	—	36	18
300	—	56	19
400	—	51	13
100	6	6	6
200	6	11	6
300	6	18	6
400	6	18	5

the iron solution contains a sulphate concentration about equal to the concentration of sulphate in the solutions in question. (Table 2.) The experiments were made on a column of 20 ml Dowex 2, 0.2—0.4 mm, in sulphate form. The flow rate was 4—5 ml/min. The iron was adsorbed from solutions with a volume of 60 ml, containing sulphuric acid to establish a pH of 2, different amounts of iron(III) sulphate and in some cases 6 g Na_2SO_4. The results obtained are given in Table 2.

Even small amounts of iron interfere in the spectrophotometric determination of uranium using sodium hydroxide and hydrogen peroxide, as seen in Table 3.

It was found that, if the iron present is masked as hexacyanoferrate(II), the interference of the iron on the spectrophotometric determination of uranium, using the sodium hydroxide and hydrogen peroxide method, is eliminated in the case of small amounts of iron and diminished in the case of larger

Table 3. Spectrophotometric determinations of uranium in solutions containing sodium hydroxide, hydrogen peroxide and varying amounts of iron. The determinations were made with a Beckman spectrophotometer model B at 3 900 Å using 5 cm cells.

Added to 50 ml solution mg U	mg Fe	U found mg	Difference %
2.14	0.05	2.23	+ 4.4
2.14	0.10	2.33	+ 8.8
2.14	0.10	2.29	+ 6.8
2.14	0.15	2.35	+ 9.9
2.14	0.20	2.42	+ 13.3
2.14	0.25	2.50	+ 16.7
2.14	0.30	2.57	+ 19.9
2.14	0.30	2.56	+ 19.7
2.14	0.45	2.82	+ 32.0
2.14	0.50	2.86	+ 33.5
2.14	0.75	3.38	+ 57.8
2.14	0.75	3.28	+ 53.3
2.14	* 2.00	3.03	+ 41.4
2.14	* 3.00	2.99	+ 39.5
2.14	* 4.00	2.96	+ 38.5

* The solution was centrifuged before the photometric determination.

Table 4. Spectrophotometric determinations of uranium from solutions containing tartaric acid, potassium cyanide, sodium hydroxide, hydrogen peroxide and varying amounts of iron. The determinations were made with a Beckman spectrophotometer model B at 3 900 Å using 5 cm cells.

Added to 50 ml solution mg U	mg Fe	U found mg	Difference %
2.34	0.4	2.34	± 0
2.34	0.6	2.35	+ 0.4
2.34	0.8	2.36	+ 0.9
2.34	1.0	2.36	+ 0.9
2.34	1.2	2.38	+ 1.7
2.34	1.4	2.39	+ 2.1
2.34	1.6	2.37	+ 1.3
2.34	1.8	2.39	+ 2.1
2.34	2.0	2.39	+ 2.1
2.50	0.4	2.50	± 0
2.50	0.6	2.50	± 0
2.50	0.8	2.51	+ 0.4
2.50	1.0	2.51	+ 0.4
2.50	1.2	2.53	+ 1.2
2.50	1.4	2.53	+ 1.2
2.50	1.6	2.54	+ 1.6
2.50	1.8	2.54	+ 1.6
2.50	2.0	2.55	+ 2.0
18.95	4	19.10	+ 0.8
18.95	7	19.03	+ 0.4
18.95	10	19.22	+ 1.4
18.95	13	19.35	+ 2.1
18.95	15	19.78	+ 4.4
18.95	18	19.86	+ 4.8

amounts of iron. Experiments were carried out to test the masking effect. Tartaric acid was first added to solutions containing hexavalent uranium and varying amounts of trivalent iron, followed by additions of potassium cyanide, sodium hydroxide and hydrogen peroxide. The latter reduces the hexacyanoferrate(III) to hexacyanoferrate(II). Results are to be found in Table 4.

EXPERIMENTAL

The apparatus used for the separation of uranium is of a common type. It can accommodate 5 ml of the ion exchange resin. The diameter of the column is 10 mm.

Aqueous solutions

Hydrochloric acid:	1 : 10	
Sodium sulphate:	10 %	
Sulphosalicylic acid:	10 %	
Sodium dithionite (hyposulphite):	2.5 %	
Sodium hydroxide:	50 %	
Sodium hydroxide:	5 %	
Iodine:	0.1 N	containing 25 g KI per litre
Tartaric acid:	25 %	
Potassium cyanide:	20 %	
Hydrogen peroxide:	4 %	

Procedure

The ion exchanger consisting of 5 ml Dowex 2, grain size 0.1—0.2 mm, is washed with HCl 1 : 10 until 45 ml of the effluent gives a blank only slightly higher than that obtained from 45 ml of the acid itself. The ion exchanger is then transformed into the sulphate form by washing with 25 ml of the sodium sulphate solution.

The solution taken for an analysis may contain 1—20 mg of uranium. Its volume ought not to exceed 50 ml and the stoichiometric concentration of SO_4 ought not to exceed 75 mg per ml.

The pH of the test solution is adjusted to 1—1.5 and thereafter 0.25 ml of sulphosalicylic acid is added. The solution is heated to about 40° C and sodium dithionite is carefully added until the red colour disappears. Another suitable reducing agent can alternatively be used. The solution is boiled 2—3 minutes and allowed to stand 5 minutes to cool. Four ml of the iodine solution is then added to ensure that the uranium is quantitatively present in the hexavalent state. The small percentage of iron simultaneously reoxidized does practically not interfere.

The solution is boiled for 1—2 minutes to expel the excess of iodine and cooled. The pH is then adjusted to 1.9—2.1 with 5 % sodium hydroxide. If the concentration of sulphate exceeds the above-mentioned value, an appropriate volume of water is added. The solution is now passed through the anion exchanger. After complete percolation the ion exchanger is washed twice with 25 ml water. The uranium is then eluted with 45 ml hydrochloric acid. When performing routine analyses it is practical to work with about twenty ion exchanger columns simultaneously.

If a rapid determination is desired the percolation and the washing can be accelerated by suction, which must be so regulated that the time of percolation is not shorter than 5 minutes. The elution must be carried out without suction.

The solution containing the eluted uranium is evaporated to 20—25 ml. In turn 2 ml tartaric acid, 2 ml potassium cyanide, 6 ml 50 % sodium hydroxide and 3 ml hydrogen peroxide are mixed with the solution. The mixture is then heated till bubbles of oxygen appear, cooled to room temperature, transferred to a 50 ml measuring flask and diluted to the mark with distilled water.

Table 5. Comparison of results of uranium determinations in test solutions according to the ion exchange method and the continuous ether extraction method.

Uranium added mg	Ion exchange method Uranium found mg	Difference %	Extraction method Uranium found mg	Difference %
7.27	7.24	− 0.4	7.08	− 2.6
7.27	7.18	− 1.2	7.08	− 2.6
7.27	7.27	± 0.0	7.15	− 1.7
7.27	7.12	− 2.2	7.13	− 1.9
7.27	7.28	+ 0.1	7.12	− 2.1
7.27	7.23	− 0.6	7.15	− 1.7
7.27	7.18	− 1.2	—	—
7.27	7.33	+ 0.8	—	—
7.27	7.24	− 0.4	—	—
7.27	7.24	− 0.4	—	—
0.727	0.730	+ 0.4	0.742	+ 2.1
0.727	0.790	+ 8.7	0.727	± 0.0
0.727	0.768	+ 5.6	0.663	− 8.8
0.727	0.790	+ 8.7	0.685	− 5.8
0.727	0.768	+ 5.6	0.663	− 8.8
0.727	0.830	+ 14.2	0.685	− 5.8
0.727	0.730	+ 0.4	0.709	− 2.5
0.727	0.790	+ 8.7	0.682	− 6.2

Table 6. Comparison of results of uranium determinations by the ion exchange method and the continuous ether extraction method.

Uranium concentration found	
Ion exchange method g/l	Extraction method g/l
0.006	0.006
0.026	0.022
0.034	0.032
0.054	0.053
0.069	0.068
0.116	0.119
0.148	0.141
0.184	0.181
0.196	0.193
0.291	0.287
0.296	0.289
0.306	0.302
0.364	0.363
0.370	0.360
0.414	0.406
0.457	0.450
2.63	2.58
2.83	2.77

In a Beckman spectrophotometer model B the extinction at 3 900 Å is determined for this solution.

Of the hydrochloric acid 45 ml are taken and evaporated to 20—25 ml and treated in exactly the same manner as the test solution. The resulting solution is used as reference in the spectrophotometric determination of uranium.

The method was tested on two especially prepared solutions, and the results compared with the results obtained according to a continuous ether extraction method [2] combined with the above spectrophotometric method of determining uranium. One litre of each solution contained 15.5 g Fe^{2+}, 1.5 g Fe^{3+}, 11 g Al, 5 g Mg, 3.5 g K, 0.1 g V and 0.005 g Cr, all as sulphates, together with 55 g H_2SO_4 and 6 g Na_2HPO_4. The solutions thus contained a stoichiometric concentration of 170 g/l SO_4. One solution contained 0.290 g/l uranium(VI) as sulphate and the other 0.0145 g/l uranium(VI) as sulphate. The results obtained are given in Table 5.

From the first solution 25 ml were taken for every analysis, corresponding to 7.27 mg U. The ion exchange method gave on the average an error of —0.6 % and the continuous ether extraction method —2.1 %.

From the second solution 50 ml were taken for every analysis corresponding to 0.727 mg U. The ion exchange method showed on the average a positive error, namely + 6.5 %. On the other hand the continuous ether extraction method yielded on the average a negative error, —4.8 %.

In the latter case, where the concentration of iron was 17 g/l and that of uranium 0.0145 g/l, there were 1 170 times as much iron as uranium in the solution, and the amount of iron adsorbed by the ion exchanger was larger

than the total amount of uranium in the solution taken for an analysis. The ratio between the concentrations of iron and uranium in the eluate would thus be unfavourable for the spectrophotometric determination of uranium *(vide supra)*, and positive errors were to be expected.

A number of solutions, *e.g.* obtained at research work, containing different concentrations of uranium, was analysed by the ion exchange method and by the continuous ether extraction method combined with the spectrophotometric determination of uranium. Table 6 shows some results.

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The Effect of Substituents and α,β-Unsaturation on the Alkaline Hydrolysis of α,β-Unsaturated Aliphatic Esters

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The alkaline hydrolysis of the ethyl esters of propiolic, tetrolic and *tert.* butylpropiolic acids has been studied in the temperature range 0—40° C in water (only the first two esters) and acetone-water mixtures. The rate constants of the alkaline hydrolysis of the first two esters in water at 25° C are 4.68 and 0.568 l mole^{-1}sec.$^{-1}$ and those for the three esters in 50 wt.% acetone-water 1.302, 0.1139 and 0.0481, respectively. The activation energies for these esters are approximately 1 500 cal. higher than those of the corresponding saturated esters, and hence the approximately fifty times higher rates of the former esters are due to high values of the Arrhenius frequency factor. By comparing the rates of the alkaline hydrolysis of various saturated, α,β-ethylenic and α,β-acetylenic esters, it has been concluded that the effects of substituents in the latter two groups of esters are primarily due to conjugative effects which lead to the higher differences in the rates of the unsaturated esters compared with those of the corresponding saturated esters.

The unsaturated bonds in α,β-unsaturated esters may be expected to effect a pronounced increase in the rate of alkaline hydrolysis of these esters owing to their electronegativity but a decrease in this rate by virtue of conjugation with the carboxyl carbonyl group. In addition, substituents at the outer end of the unsaturated bond may influence the rate by an inductive, conjugative or hyperconjugative effect. Since it is not possible to distinguish between these effects by a study of the rates of alkaline hydrolysis of α,β-ethylenic and saturated aliphatic esters alone, the alkaline hydrolysis of several α,β-acetylenic esters has been investigated to obtain more information on the interplay of these various factors.

EXPERIMENTAL

Ethyl propiolate. 16 g of propiolic acid prepared as described previously [1] were dissolved in 60 ml of absolute alcohol containing 5 g of concentrated sulphuric acid. After the mixture had stood at room temperature for 60 hours, water was added and the ester formed extracted with ether. The ether solution was washed with sodium bicarbonate

solution and dried with calcium chloride, after which the solvent was evaporated. The ester was distilled by fractionation in a Widmer column. Its b.p. was 120.0° C at 760 mm Hg. The yield was 9.15 g (40 % of theory). The ester was a clear liquid with a pungent odour which strongly irritated the eyes and skin.

Ethyl tetrolate. This ester was prepared from previously synthesized tetrolic acid [1] by the preceding method except that the reaction mixture was heated on the water bath for one hour. The ester boiled at 164.5—165.0° at 760 mm Hg. The yield was 64 % of the theoretical. The odour of the ester resembled that of ethyl propiolate, but was less intense and less disagreeable.

Ethyl t e r t. *butylpropiolate.* This ester was obtained by esterification of previously prepared *tert.* butylpropiolic acid [2] according to the method given above for ethyl propiolate, but with the difference that the rection mixture was boiled two hours on the water bath and the ester distilled under diminished pressure. B. p. 83.0—83.5° C at 23 mm Hg. The yield was 95 % of the theoretical. The ester had a weak pleasant odour.

Total saponification tests conducted on these esters gave a purity of 100 % within the limits of experimental accuracy, in view of which they were sufficiently pure for kinetic measurements.

Acetone (pro analysi, Merck) and sodium hydroxide (reagent grade, Baker) were used as such. The water employed was ordinary distilled water.

Kinetic measurements. The hydrolyses were conducted in three-necked reaction flasks[3]. Seven samples were titrated in each experimental run at each temperature. The concentrations of sodium hydroxide and ester were both 0.01 *M* in the initial mixture and the sample volume 10 ml. After appropriate intervals, the reaction was arrested by adding an excess of 0.02 *N* hydrochloric acid, and the excess of acid was titrated with 0.02 *N* barium hydroxide using cresol red as indicator.

From the total saponification values it was concluded that the triple bonds do not add water at the temperatures employed. The esters did not hydrolyse in water or dilute hydrochloric acid during several days at room temperature.

RESULTS

The experimental data are collected in Table 1. The rate constants have been computed from the usual second order rate equation. The activation energies and frequency factors were calculated by the method of least squares. The rate constants were found to decrease approximately 5 % in the experimental run when the reaction progressed from 20 to 70 % saponification. The decrease in the values of the rate constants increased with temperature and with the water content of the solvent. Since the total saponification gave an approximately 100 % reaction, the decrease in the rate constant is evidently due to the evaporation of the ester into the gas phase in the reaction flask.

Table 1. Kinetic data on the alkaline hydrolysis of ethyl propiolate, tetrolate, and t e r t. *butylpropiolate in water and in acetone-water mixtures.*

Reactant	Solvent composition wt % H_2O	k l mole^{-1} sec.$^{-1}$					E cal.	log A
		0°	13.00°	15.00°	32.00°	40.00°		
$HC \equiv C \cdot CO_2Et$	100	0.661	1.93	4.68	7.33	11.7	12 260	9.638
—»—	80	0.637	1.62	3.54	5.13	8.06	10 780	8.435
—»—	50	0.235	0.572	1.30	2.09	3.35	11 360	8.448
$CH_3 \cdot C \equiv C \cdot CO_2Et$	100	0.0761	0.229	0.568	0.901	1.51	12 710	9.055
—»—	80	0.0633	0.159	0.355	0.545	0.859	11 120	7.699
—»—	50	0.0178	0.0475	0.114	0.182	0.298	12 030	7.868
$(CH_3)_3C \cdot C \equiv C \cdot CO_2Et$	50	0.00716	0.0197	0.0481	0.0808	0.138	12 590	7.920

DISCUSSION

In Table 2 are given the specific rates at 25° C, the activation energies and frequency factors for the alkaline hydrolysis of the ethyl esters of the α,β-acetylenic (excluding *tert.* butylpropiolic ester since the corresponding saturated and α,β-ethylenic esters have not been studied kinetically), α,β-ethylenic acids and corresponding saturated acids in aqueous solution.

Table 2. The rate constants, activation energies and frequency factors for the alkaline hydrolysis of the ethyl esters of some saturated and α,β-unsaturated acids in water.

Reactant	$k_{25°}$	E cal.	log A
$H_2C \cdot CH_2CO_2Et$ [4]	0.0870	10 720	6.780
$H_2C \cdot CH_2 \cdot CH_2CO_2Et$ [4]	0.0383	10 320	6.142
$^-OOC \cdot CH_2 \cdot CH_2CO_2Et$ [5]	0.0177	11 050	6.35
$EtO_2C \cdot CH_2 \cdot CH_2CO_2Et$ [5]	0.207	11 240	7.55
trans:			
$H_2C = CH \cdot CO_2Et$ [6]	0.0779	11 950	7.638
$H_2C \cdot HC = CH \cdot CO_2Et$ [6]	0.0130	12 720	7.431
$^-OOC \cdot HC = CH \cdot CO_2Et$ [6]	0.158	11 120	7.375
$EtO_2C \cdot HC = CH \cdot CO_2Et$ [6]	4.47	11 600	9.159
cis:			
$H_2C = CH \cdot CO_2Et$ [6]	0.0779	11 950	7.638
$H_2C \cdot HC = CH \cdot CO_2Et$ [7]	0.0146	—	—
$^-OOC \cdot HC = CH \cdot CO_2Et$ [6]	0.00486	13 390	7.502
$EtO_2C \cdot HC = CH \cdot CO_2Et$ [6]	0.407	11 500	8.064
$HC \equiv C \cdot CO_2Et$	4.68	12 260	9.638
$H_2C \cdot C \equiv C \cdot CO_2Et$	0.568	12 705	9.055
$^-OOC \cdot C \equiv C \cdot CO_2Et$ [8]	2.40	11 380	8.71
$EtO_2C \cdot C \equiv C \cdot CO_2Et$ [8]	69.0	10 900	9.84

It will be noted that the rates of the α,β-acetylenic esters are much higher than the rates of the corresponding α,β-ethylenic and saturated esters. As compared with the propionic ester, the acrylic and propiolic esters are characterized by a $-I$ effect of the unsaturated bond and conjugation between the latter and the carboxyl carbonyl group. Although conjugation is not probably very marked in unsaturated acids [9], it is much greater in their esters since the conjugation energy of the carbalkoxyl group is approximately 4 000 cal. smaller than that of the carboxyl group in the acid [10]. In the acrylic ester the decrease in the rate caused by the conjugation overweighs the weak rate-promoting inductive effect of the sp^2 valence type of the double bond, and consequently the rate of this ester is smaller than that of the propionic ester. This conjugation is of the same magnitude in both propiolic and acrylic esters since only one p-electron pair can conjugate with a carbonyl group. In the triple bond the valence type is sp and accordingly its inductive effect is approximately a hundred times as great as that of a double bond. It is, however, surprising that although, as expected, the rate of the propiolic ester is nearly

60 times as great as the rates of the propionic and acrylic esters, the activation energy for the former ester is about 1 500 cal. higher than that of the propionic ester and of the same magnitude as that of the acrylic ester. (The energy difference is perhaps too great if, as suggested by Tommila [4], hydrogen bonding between the terminal methyl group and the carbonyl oxygen contributes to the activation energy in case of hydrolysis of the saturated esters). Hence it is not even approximately true that the activation energy of the ester is proportional to the dissociation constant of the corresponding acid, since the dissociation constant of propionic acid at 25° C is 1.33×10^{-5}, that of acrylic acid 5.56×10^{-5} and that of propiolic acid about 500×10^{-5}. On the other hand, the activation energy appears as a rule to be proportional to the dissociation constant in a homologous series of acids, but this is no longer true for several series of acids. Since the activation energies for the propiolic and acrylic esters are nearly equal, their higher activation energy as compared with that of the propionic ester is evidently due to the conjugation between the unsaturated bond and the carboxyl carbonyl group, but the observed difference in the activation energies cannot readily be explained on the basis of generally accepted views on the mechanisms of alkaline ester hydrolysis [9,11].

In the series of butyric, *trans*-crotonic and tetrolic esters, the methyl group introduces a $+I$ effect and in the crotonic ester there is π_y-hyperconjugation and in tetrolic ester a π_y- and π_z-hyperconjugation. From Table 1 we see that the rates of the ethyl esters of propiolic, tetrolic and *tert.* butylpropiolic acids in 50 % acetone-water at 25° C are 1.30, 0.114 and 0.0481 1 mole^{-1} sec^{-1}. The ratios of these rates are about 11 and 2.4, which indicates that the difference in the rates for the propiolic and tetrolic esters is due mainly to hyperconjugation. Hyperconjugation does not naturally exert as great a retarding effect on the alkaline hydrolysis of tetrolic ester as on the decarboxylation of tetrolic acid [12], since it acts partly inductively in the former case. The ratio of the rates of hydrolysis of the propiolic and tetrolic esters is about 8 in water and about 11 in 50 % acetone-water at 25° C while the ratio of the decarboxylation rates of the anions of the acids in highly non-polar solvents is approximately 34 at 106.78° C. A similar view of the effect of the methyl group on the rates of the alkaline hydrolysis of α,β-unsaturated esters is obtained when the ratios of the rates of the following pairs of esters are compared: propionic-butyric, acrylic-*trans*crotonic and propiolic-tetrolic; the ratios are in water at 25° C 2.27, 5.99 and 8.23, respectively. The ratios thus increase with the degree of hyperconjugation. If the inductive effect alone determined the reaction rates, no greater differences would be observed between the ratios. The last ratio is smaller than would be expected if the effects were additive, but it seems natural that the effect of hyperconjugation ($+I$) on the reaction rate is relatively smaller in relation to the strong inductive effect of the triple bond than to the weak inductive effect of the double bond. Since the rate of hydrolysis of the ethyl ester of *cis*-crotonic acid is almost as large as that of the *trans*-crotonic ester, although conjugation presumably is not possible in the former, it must be concluded that a strong steric effect operates in the *cis*-crotonic ester.

The carboxylate ion group is electropositive by nature [9,12], also when it is attached to an unsaturated bond, since the internal resonance, about 36 000

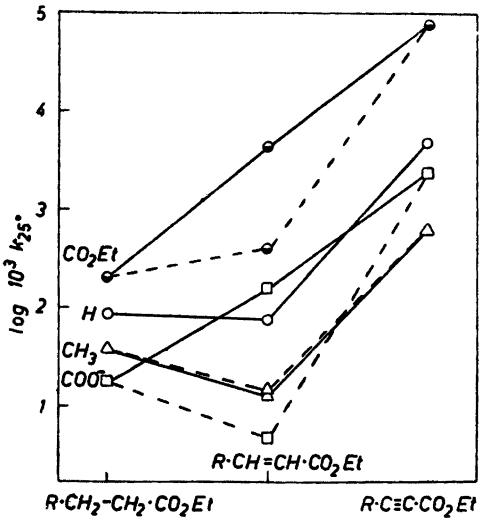

Fig. 1. The logarithm of the rate constant as a function of bond order for the alkaline hydrolysis of the ethyl esters of some saturated and α,β-unsaturated acids in water. The points joined by broken lines refer to esters of cis acid.

cal.[10], of the group is so large that it does not enter into conjugation with a neighbouring unsaturated bond. However, the carboxylate ion group is strongly solvated in water, the solvation energy being of the order of 6 000 cal.[13]. Consequently the negative charge is masked, as a result of which the inductive effect becomes more electronegative and also conjugation is favoured. The effect of the ionised carboxylate group in the ester hydrolysis of monoethyl succinate and monoethyl acetylenedicarboxylate (Table 2, Fig. 1) acts in the expected direction, *i. e.* the rates are smaller than those of the propionic and propiolic esters. The rate of hydrolysis of the fumaric ester ion, on the other hand, is about twice that of ethyl acrylate in water, but the ratio decreases when an organic solvent is added to the solvent, being 1.26 in 58.3 % ethanol-water, and it is possible that the ratio will decrease below unity as the solvent becomes less polar. Also the change in the activation energy with the composition of solvent in ethanol-water and dioxan-water mixtures is greater for the fumaric ester ion than for diethyl fumarate [6], which may also be due to the solvation of the carboxylate ion group. Also the reaction rates, activation energies and frequency factors of the *m*- and *p*-phthalic ester ions are approximately equal, the rates in water being approximately twice that of ethyl benzoate [14].

The rates of hydrolysis of the diethyl esters of fumaric and acetylenedicarboxylic acids are clearly greater than the rate of diethyl succinate. In unsaturated esters the main effect is the conjugative effect since the ratios of the rates of hydrolysis of esters of the acid pairs, succinic-propionic, fumaric-acrylic and acetylene-dicarboxylic-propiolic are 2, 60 and 15. A similar weakening as that observed in the case of the hyperconjugative effect is noted in the conjugative effect in the acetylenic compounds as compared to the ethylenic compounds. If we compare the ratios of the rates for the ester pairs, diethyl- and monoethyl succinates, diethyl- and monoethyl fumarates and diethyl-

and monoethyl acetylenedicarboxylates, 11.7, 28.3 and 28.7, we find that these are exactly in accordance with the assumed variation of the conjugative effect. From Fig. 1 it will be seen that owing to conjugative effects of the substituents, larger differences exist between the rates of the esters of α,β-unsaturated esters than between the rates of saturated esters.

The solvent effect is normal for all esters studied [15,4] the rate of alkaline hydrolysis decreases as the acetone content increases in acetone-water mixtures (Table 1), and a minimum in the activation energy, a depression of about 1 500 cal., is observed when the mixture contains approximately 20 % acetone.

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The Reaction of Hydrazine with Cinnamic Acid Derivatives

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The product formed by reaction of ethyl cinnamate with one mole equivalent of hydrazine hydrate is shown to be 3-phenyl-5-pyrazolidone and not as previously assumed cinnamic acid hydrazide.

Of the two products, formed upon treatment of ethyl *m*-nitrocinnamate with hydrazine hydrate, the main product (m. p. 139°) is shown to be 3-(*m*-nitrophenyl)-5-pyrazolidone, while the by-product (m. p. 198°) is *m*-nitrocinnamic acid hydrazide.

The two pyrazolidones mentioned are able to condense with benzaldehyde forming benzylidene compounds to which betain formulas are proposed.

A method of synthesis of α,β-olefinic acid hydrazides is given, consisting in treatment of the mixed anhydride of the appropriate α,β-olefinic acid and monoethylcarbonic acid with hydrazine hydrate. By this method cinnamic acid hydrazide, *m*-nitrocinnamic acid hydrazide and *p*-nitrocinnamic acid hydrazide have been synthesized.

Finally it is shown, that treatment of ethyl cinnamate with two mole equivalents of hydrazine hydrate leads to the formation of β-hydrazinodihydrocinnamic acid hydrazide.

The literature dealing with the reaction of the esters of α,β-olefinic acids with hydrazine reveals that some confusion prevails with regard to this subject.

When treating ethyl cinnamate with hydrazine hydrate, Muckermann [1] obtained a substance with m. p. 101°, which he assumed to be cinnamic acid hydrazide. This substance has the characteristic properties of a primary hydrazide, with the exception that it does not, as was to be expected, form cinnamoyl azide with nitrous acid, but 2-nitroso-3-phenyl-5-pyrazolidone. This reaction Muckermann explained to be due to cyclization upon treatment with nitrous acid. According to the same author, ethyl crotonate reacts similarly.

Upon treatment of ethyl *m*-nitrocinnamate with hydrazine hydrate, Curtius and Bleicher [2] were able to isolate two isomeric compounds; both were assumed to be *m*-nitrocinnamic acid hydrazides. The main product (m. p. 139°) formed a nitrosopyrazolidone with nitrous acid, while the by-product (m. p. 198°) reacted normally, forming *m*-nitrocinnamic acid azide (Curtius

and Kengott [3]). To both substances, however, the hydrazide structure was assigned, on the ground that they condensed with aldehydes and ketones.

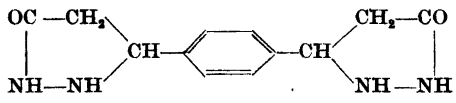
Gansser and Rumpf [4] treated methyl *p*-nitrocinnamate with hydrazine hydrate and obtained as the main product *β*-(*p*-nitrophenyl)-*β*-hydrazinopropionic acid hydrazide and, in addition, minute quantities of a substance which was presumed to be *p*-nitrocinnamic acid hydrazide.

By causing ethyl metacrylate to react with anhydrous hydrazine, Lieser and Kemner [5] obtained a substance which was assumed to be 4-methyl-5-pyrazolidone, because it formed 2-nitroso-4-methyl-5-pyrazolidone with nitrous acid.

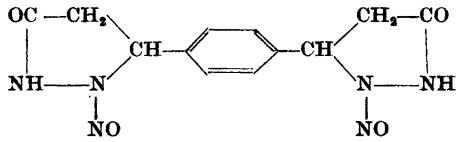
By reaction of *p*-phenylenediacrylic acid diethyl ester with hydrazine hydrate in alcohol Ruggli and Theilheimer [6] obtained a substance which, upon treatment with nitrous acid, did not give the expected azide, but the nitroso-pyrazolidone (c). The authors discuss whether the substance has the structure (a) or (b), but they favour formula (a).

(a) $H_2N-NH-CO-CH=CH-C_6H_4-CH=CH-CO-NH-NH_2$

(b)



(c)



Freri [7] has synthesized the dihydrazides of itaconic acid, mesaconic acid, and citraconic acid from the corresponding methyl esters by reaction with hydrazine hydrate in alcohol. With nitrous acid all these hydrazides form azides in the normal manner. According to Curtius [8], diethyl fumarate behaves similarly.

In an investigation into the reaction of hydrazine hydrate with lactones, Darapsky [9] brought the unsaturated lactone, coumarin, to react with hydrazine hydrate in alcohol. He did not obtain *o*-coumaric acid hydrazide, but *β*-hydrazino-*o*-dihydrocoumaric acid hydrazide which, upon treatment with nitrous acid, formed 2-nitroso-3-(*o*-hydroxyphenyl)-5-pyrazolidone.

A similar observation has been made by Stodola [10] who, in an attempt to synthesize *α*-benzoylaminocinnamic acid hydrazide from the corresponding methyl ester by reaction with hydrazine hydrate in methanol, obtained *α*-benzoylamino-*β*-hydrazinodihydrocinnamic acid hydrazide which, upon heating *in vacuo*, split off hydrazine with the formation of 3-phenyl-4-benzoylamino-5-pyrazolidone. The structure of this compound was established from the ultraviolet absorption spectrum and the reaction with nitrous acid, whereby 2-nitroso-3-phenyl-4-benzoylamino-5-pyrazolidone is formed.

We have chosen cinnamic acid as the object of our investigation since it is one of the simplest and most thoroughly investigated representatives of *α*,*β*-olefinic acids.

It has been possible to show that Muckermann's "cinnamic acid hydrazide" with m. p. 101° (which, as previously mentioned, is formed when ethyl cinnamate is treated with a small excess of hydrazine hydrate in alcohol) is not cinnamic acid hydrazide. In addition to the abnormal reaction with nitrous acid, the following properties are in disagreement with the hydrazide structure:

1) The ultraviolet absorption spectrum does not show the peak at 2 800 Å, characteristic of the system

$$C_6H_5—C=C—C=O$$

2) It has not been possible to hydrogenate the compound catalytically with a platinum oxide catalyst to dihydrocinnamic acid hydrazide.

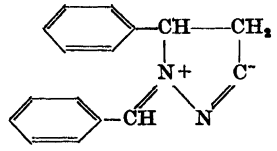
We therefore made an attempt at synthesizing cinnamic acid hydrazide by bringing the mixed anhydride of cinnamic acid and monoethylcarbonic acid (I) to react with hydrazine hydrate *. Hereby a substance is formed in about 65 % yield and m. p. 117—117.5°, which is isomeric with Muckermann's compound, the properties of which correspond, however, in every respect to those expected for cinnamic acid hydrazide (II):

1) With nitrous acid cinnamic acid azide (III) is formed.
2) The ultraviolet absorption spectrum (curve 2, Fig. 1) shows a strong absorption maximum at 2 750 Å.
3) Upon catalytic hydrogenation with a platinum catalyst dihydrocinnamic acid hydrazide (IV) is obtained.

Muckermann's compound must consequently be considered as 3-phenyl-5-pyrazolidone (V), a structure which is further evidenced by the fact that we have been able to oxidize the substance to 3-phenyl-5-pyrazolone (VI).

The fact that the substance condenses with aldehydes and ketones seems inconsistent with the pyrazolidone formula, and this is presumably the reason why Muckermann assumed the substance to be cinnamic acid hydrazide.

This property can be explained, however, if betain structures are assigned to the condensation products. The benzylidene compound, for example, can be described by formula VII.



VII

In accordance with this formulation, the absorption spectrum (curve 3, Fig. 1) shows two peaks at 3 300 Å and 3 470 Å, respectively, and moreover the substance is readily hydrolyzed in acid solution. In this connection, it is worth mentioning Pugh's [14] recent observation that condensation of acetone with 3,5,5-trimethyl-Δ^2-pyrazoline in the presence of acids leads to the formation of 3,5,5-trimethyl-1-*iso*propylidenepyrazolinium salts.

* The application of ethyl chlorocarbonate to this problem suggested itself in view of the recent use of this compound in peptide syntheses [11-13].

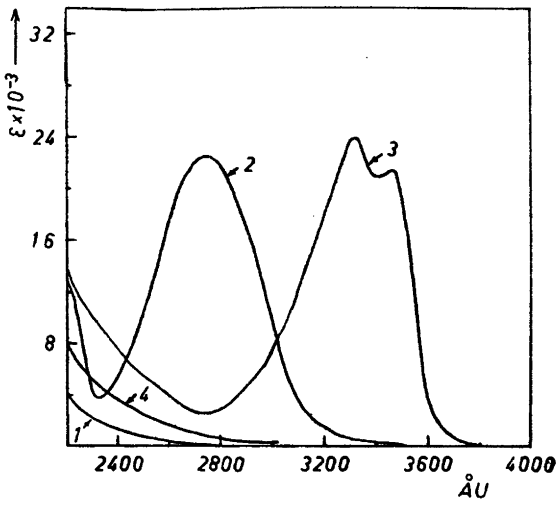


Fig. 1. Ultraviolet absorption spectra.

Curve 1. 3-Phenyl-5-pyrazolidone.
Curve 2. Cinnamic acid hydrazide.
Curve 3. Benzylidene compound of 3-phenyl-5-pyrazolidone.
Curve 4. 2-Benzyl-3-phenyl-5-pyrazolidone.
ε = molar extinction coefficient (conc. in moles/l.)

Upon catalytical hydrogenation of the benzylidene compound in the presence of a platinum oxide catalyst one mole equivalent of hydrogen is absorbed.

The reaction product which melts at 114.5—115° has amphoteric properties and must be considered to be 2-benzyl-3-phenyl-5-pyrazolidone (VIII).

In agreement with this formula, the absorption spectrum (curve 4, Fig. 1) shows no peak at 2 800 Å.

When a palladium catalyst was substituted for platinum in the hydrogenation, the reduction proceeded further under ring opening; when the hydrogenation was stopped as soon as two mole equivalents of hydrogen had been absorbed, dihydrocinnamic acid benzylhydrazide (IX) was obtained. The latter compound was also obtained by hydrogenation of benzylidene cinnamic acid hydrazide (X) over platinum.

Continued hydrogenation of VII over palladium on charcoal causes the uptake of three mole equivalents of hydrogen because the dihydrocinnamic acid benzylhydrazide (IX) is debenzylated and dihydrocinnamic acid hydrazide (IV) is formed.

When refluxing ethyl cinnamate in ethanol with approximately three mole equivalents of hydrazine hydrate, 3-phenyl-5-pyrazolidone is not obtained; the crystalline product (m. p. 103—105°) turned out to be β-hydrazinodihydrocinnamic acid hydrazide (XI).

With two equivalents of benzaldehyde this substance forms the dibenzylidene compound (XII).

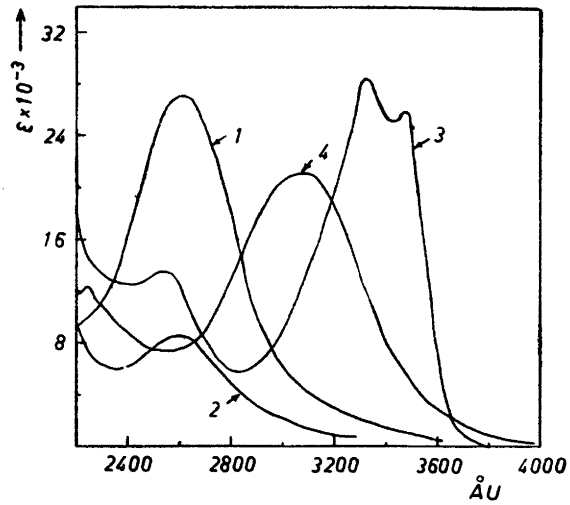


Fig. 2. Ultraviolet absorption spectra.

Curve 1. m-*Nitrocinnamic acid hydrazide.*
Curve 2. *3-(*m-*Nitrophenyl)-5-pyrazolidone.*
Curve 3. *Benzylidene compound of 3-(*m-*Nitrophenyl)-5-pyrazolidone.*
Curve 4. p-*Nitrocinnamic acid hydrazide.*
ε = molar extinction coefficient (conc. in moles/l.)

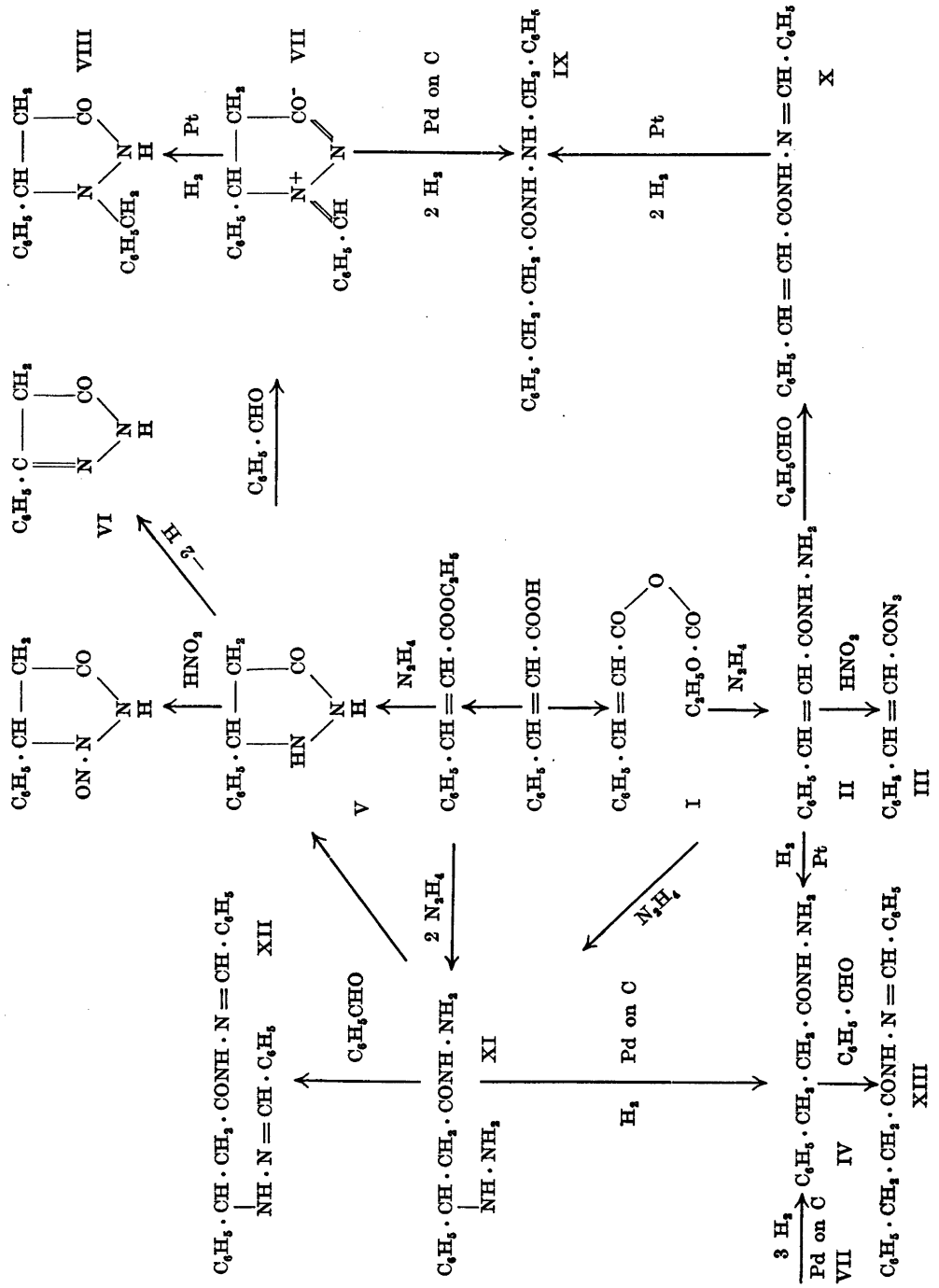
During distillation at reduced pressure β-hydrazinodihydrocinnamic acid hydrazide (XI) splits off hydrazine and forms 3-phenyl-5-pyrazolidone (V), and by catalytical hydrogenation over Pd on charcoal dihydrocinnamic acid hydrazide (IV) is formed.

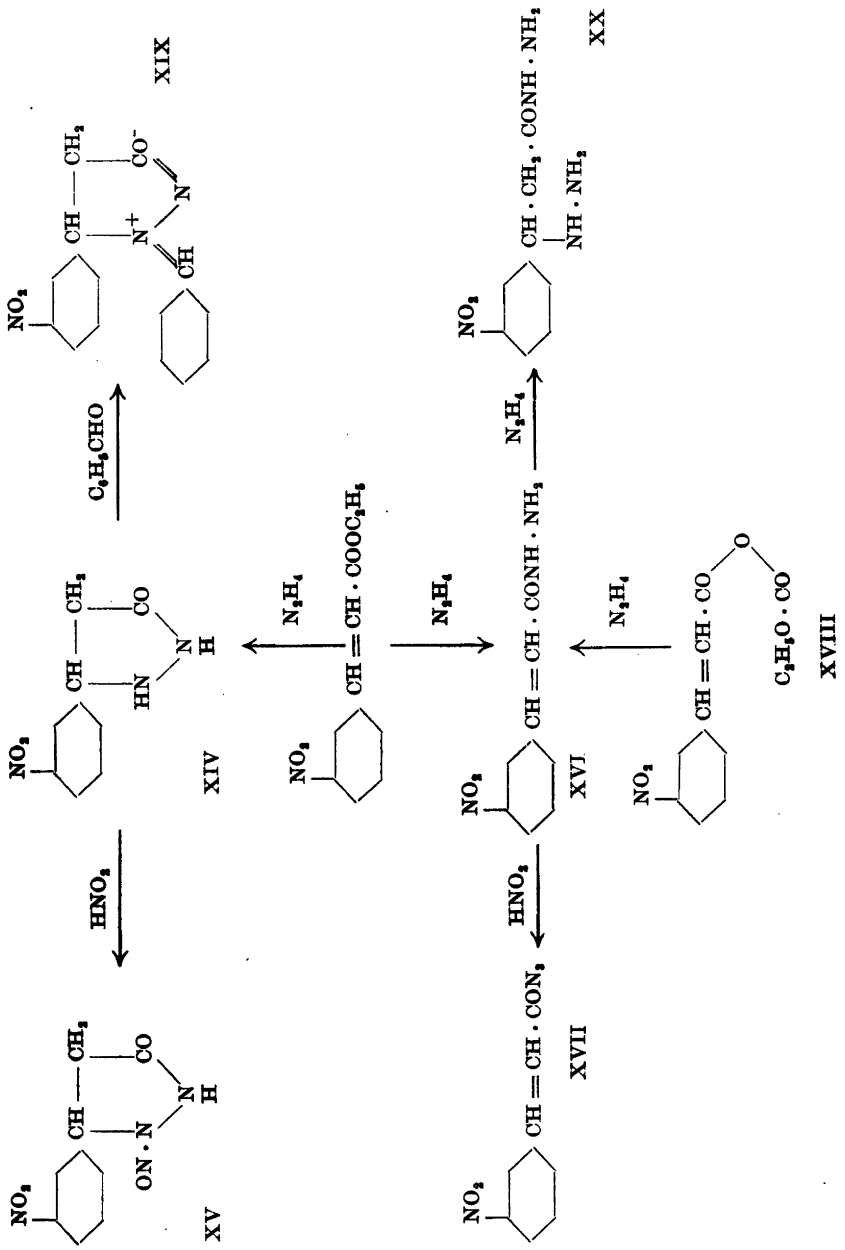
As we were unable to convert cinnamic acid hydrazide into 3-phenyl-5-pyrazolidone, it is assumed that the first step in the formation of the latter compound is an addition of hydrazine to the double bond in ethyl cinnamate followed by cyclization of the ethyl β-hydrazinodihydrocinnamate formed during this reaction.

The cyclization, however, is a relatively slow process, and in the presence of an excess of hydrazine the latter reacts with the hydrazinoester forming β-hydrazinodihydrocinnamic acid hydrazide. As recently shown by Phillips and Mentha [15], the reaction of methyl cinnamate with guanidine takes place in a similar way.

From the results stated above it might be concluded that Curtius' "*m*-nitrocinnamic acid hydrazide" (m. p. 139°) which, upon treatment with nitrous acid, yields 2-nitroso-3-*m*-nitrophenyl-5-pyrazolidone (XV) is 3-*m*-nitrophenyl-5-pyrazolidone (XIV) and that the substance (m. p. 199°) which normally forms *m*-nitrocinnamoyl azide (XVII) actually is *m*-nitrocinnamic acid hydrazide (XVI).

This assumption has been confirmed, since the ultraviolet absorption spectrum of the compound which melts at 199° shows a strong absorption maxi-





mum at the same wavelength (2 600 Å) as does *m*-nitrocinnamic acid, while the compound with m. p. 139° only absorbs weakly in this region (curve 1 and 2, Fig. 2).

Furthermore, it has been possible to synthesize the compound with m. p. 199° by causing the mixed anhydride of *m*-nitrocinnamic acid and monoethylcarbonic acid (XVII) to react with hydrazine hydrate.

In analogy to the benzylidene compound of 3-phenyl-5-pyrazolidone, the structure XIX must be assigned to the benzylidene compound of XIV, and the ultraviolet absorption spectrum is in accordance herewith (curve 3, Fig. 2).

Upon treatment of *m*-nitrocinnamic acid hydrazide (XVI) with hydrazine hydrate, β-hydrazino-*m*-nitrodihydrocinnamic acid hydrazide (XX) is obtained, although in poor yield only.

Gansser and Rumpf [4] gave no evidence of the hydrazide structure of the substance they considered to be *p*-nitrocinnamic acid hydrazide. It would therefore be of interest to establish whether this substance actually is *p*-nitrocinnamic acid hydrazide or 3-*p*-nitrophenyl-5-pyrazolidone.

To that end, we have synthesized *p*-nitrocinnamic acid hydrazide in a manner analogous to the preparation of cinnamic acid hydrazide. The melting point of the product thus obtained was not depressed by admixture with Gansser and Rumpf's compound.

The hydrazide structure of the substance is further evidenced by the ultraviolet absorption spectrum (curve 4, Fig. 2) and by the fact that treatment with nitrous acid leads to the formation of *p*-nitrocinnamoyl azide.

EXPERIMENTAL

All melting points are corrected.

Cinnamic acid hydrazide (II)

To a stirred, ice-cooled suspension of 5.58 g (0.03 mole) of potassium cinnamate in 250 ml of methylene chloride were added 3.25 g (0.03 mole) of ethylchlorocarbonate and 6 ml of a 1 % solution of pyridine in methylene chloride. Stirring was continued for two hours at 0—5° C and the reaction mixture was then slowly poured into a stirred suspension of 1.5 ml (0.03 mole) of 100 % hydrazine hydrate in 50 ml of methylene chloride. After filtration, the filtrate was kept in an ice box overnight, then washed with a saturated sodium bicarbonate solution, and dried. The solvent was removed and the residue recrystallized from benzene. Yield: 3.2 g ~ 66 %; m. p. 117—117.5° C. (Found: C 66.59; H 6.44; N 17.10. Calc. for $C_9H_{10}N_2O$: C 66.65; H 6.22; N 17.28).

Cinnamoyl azide (III)

1.62 g (0.01 mole) of cinnamic acid hydrazide (II) were dissolved in 20 ml of 4 N hydrochloric acid. The solution was cooled to +5° and an equimolar amount (690 mg) of sodium nitrite in 5 ml of water was added slowly to the stirred liquid. The white precipitate formed was removed by filtration, washed with water, and dried over phosphorus pentoxide *in vacuo*. Yield: 1.10 g ~ 64 %; m. p. 82° (decomp.).

After two recrystallizations from light petroleum (60—90°) the melting point was 85—86° (decomp.). (Found: C 62.61; H 4.19; N 24.35. Calc. for $C_9H_7N_3O$: C 62.41; H 4.07; N 24.27).

Forster [16], who has prepared the compound from cinnamoyl chloride and sodium azide reports m. p. 86° (decomp.).

Dihydrocinnamic acid hydrazide (IV)

a) *From cinnamic acid hydrazide (II).* A suspension of 1.62 g (0.01 mole) of cinnamic acid hydrazide (II) in 50 ml of absolute ethanol was shaken in the presence of 135 mg of a platinum oxide catalyst * at room temperature under one atmosphere of hydrogen. The theoretical amount of hydrogen was consumed in one hour.

The solution was filtered and concentrated *in vacuo*. Filtration gave 1.10 g ~ 66 % of white crystals which melted at 99.5—100.5° C. After recrystallization from benzene, m. p. 101—102° C. (Jordan [17] reports m. p. 103° C). (Found: C 65.81; H 7.22; N 17.26. Calc. for $C_9H_{12}N_2O$: C 65.85; H 7.37; N 17.07).

For characterization, we have prepared the benzylidene compound (XIII) as described by Jordan [17] and found m. p. 133—134° C (Jordan reports 132.5°).

b) *From β-hydrazinodihydrocinnamic acid hydrazide (XI).* 1.94 g (0.01 mole) of β-hydrazinodihydrocinnamic acid hydrazide were suspended in 100 ml of absolute ethanol and catalytically reduced at 70° under one atmosphere of hydrogen in the presence of 2.5 g of 10 % palladium-on-charcoal *.

When the theoretical amount of hydrogen had been absorbed, the catalyst was removed and the solution evaporated to dryness under reduced pressure. The residue was recrystallized from benzene. Yield 0.89 g ~ 55 %; m. p. 99—100.5° C. The melting point was not depressed by admixture with authentic dihydrocinnamic acid hydrazide.

c. *From the benzylidene compound of 3-phenyl-5-pyrazolidone (VII).* A solution of 2.50 g (0.01 mole) of the benzylidene compound of 3-phenyl-5-pyrazolidone in 100 ml of absolute ethanol was hydrogenated at room temperature under one atmosphere of hydrogen in the presence of 1.25 g of 10 % palladium-on-charcoal. When three mole equivalents of hydrogen had been absorbed, the catalyst was removed and the solution evaporated to dryness under reduced pressure. The residue was recrystallized from benzene. Yield 1.05 g ~ 64 %; m. p. 101—102° alone and by admixture with authentic dihydrocinnamic acid hydrazide.

3-Phenyl-5-pyrazolidone (V)

This compound was prepared as described by Muckermann [1] for cinnamic acid hydrazide. The yield and the melting point were in agreement with the values reported by Muckermann. The molecular weight was determined to be 168 (Rast); calc. 162.

3-Phenyl-5-pyrazolone (VI)

To a stirred ice-cooled solution of 3.24 g (0.02 mole) of 3-phenyl-5-pyrazolidone (V) in 40 ml of pyridine was added in the course of 15 minutes a solution of 6.4 g (0.04 mole) of $CuSO_4$ in 40 ml of water. After addition of 100 ml of water the precipitate which formed was filtered off, washed with water, and triturated with 15 ml of conc. hydrochloric acid. The 3-phenyl-5-pyrazolone hydrochloride which formed was filtered, washed with conc. hydrochloric acid, and dried.

The free 3-phenyl-5-pyrazolone was liberated by triturating the hydrochloride with a saturated sodium bicarbonate solution. Filtration and washing with water yielded 1.65 g ~ 51 % of air-dried product which melted at 236—244° C. After recrystallization from ethanol the melting point was 243—244°, and this was not depressed by admixture with authentic 3-phenyl-5-pyrazolone. The I.R.-spectra of the two products were identical too.

The benzylidene compound of 3-phenyl-5-pyrazolidone (VII)

This compound was prepared as described by Muckermann [1] for "benzylidene cinnamic acid hydrazide".

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* Obtained from Baker Platinum Ltd., 52 High Holborn, London, W.C. 1.

We have found it more convenient to recrystallize the compound from benzene instead of 50 % ethanol and have by this procedure obtained another modification, which melts at 200—201° C. Mol.wt. 264 (Rast); calc. 250.

2-Benzyl-3-phenyl-5-pyrazolidone (VIII)

1.00 g (0.004 mole) of the benzylidene compound of 3-phenyl-5-pyrazolidone (VII) was dissolved in 50 ml of absolute ethanol and catalytically reduced at room temperature under one atmosphere of hydrogen in the presence of 50 mg of a platinum oxide catalyst. When the theoretical amount of hydrogen had been absorbed (thirty minutes), the catalyst was removed and the solution evaporated to dryness under reduced pressure. The residue (m. p. 113.5—114° C) was recrystallized from 50 % ethanol. Yield 620 mg ~ 62 %; m. p. 114.5—115° C. (Found: C 75.94; H 6.35; N 11.20. Calc. for $C_{16}H_{16}N_2O$: C 76.17; H 6.39; N 11.11).

Dihydrocinnamic acid benzylhydrazide hydrochloride (IX)

a) *From the benzylidene compound of 3-phenyl-5-pyrazolidone (VII).* A solution of 2.50 g (0.01 mole) of the benzylidene compound of 3-phenyl-5-pyrazolidone (VII) in 150 ml of absolute ethanol was shaken in the presence of 1.25 g of 10 % palladium-on-charcoal at room temperature under one atmosphere of hydrogen. When two mole equivalents of hydrogen had been absorbed, the catalyst was removed and the solution evaporated to dryness under reduced pressure. The oily residue was dissolved in absolute ethanol and converted into the hydrochloride by addition of ethanolic hydrogen chloride followed by ether. Yield 1.50 g ~ 52 %; m. p. 163—170° C. After recrystallization from absolute ethanol the melting point was 169—171° C. (Found: C 65.83; H 6.64; N 9.71; Cl 12.63. Calc. for $C_{16}H_{19}ClN_2O$: C 66.09; H 6.59; N 9.64; Cl 12.19).

b) *From benzylidene cinnamic acid hydrazide (X).* 2.50 g (0.01 mole) of benzylidene cinnamic acid hydrazide were suspended in 50 ml of dioxan and catalytically reduced at 70° under one atmosphere of hydrogen in the presence of 125 mg of a platinum oxide catalyst. When two mole equivalents of hydrogen had been absorbed in the course of four hours, the catalyst was removed and the solvent evaporated at reduced pressure. The oily residue was worked up as described under a). Yield 1.50 g ~ 52 %; m. p. 173—75° C. (Found: C 66.13; H 7.08; N 9.51; Cl 12.24. Calc. for $C_{16}H_{19}ClN_2O$: C 66.09; H 6.59; N 9.64; Cl 12.19).

The I.R.-spectra of the two products were identical.

Benzylidene cinnamic acid hydrazide (X)

To a stirred solution of 1.62 g (0.01 mole) of cinnamic acid hydrazide in 25 ml of ethanol were added 25 ml of water, 1 ml of benzaldehyde and two drops of 4 N sulfuric acid. After 30 minutes the white precipitate formed was filtered off, washed with ethanol, and dried. Yield 2.1 g ~ 84 %; m. p. 225.5—227.5° C. Recrystallization from ethanol yielded 1.8 g which melted at 229—230° C. (Found: C 76.70; H 5.63; N 11.35. Calc. for $C_{16}H_{14}N_2O$: C 76.79; H 5.64; 11.19).

β-Hydrazinodihydrocinnamic acid hydrazide (XI)

a) *From ethyl cinnamate.* A mixture of 17.6 g (0.1 mole) of ethyl cinnamate, 18 ml (0.36 mole) of 100 % hydrazine hydrate and 10 ml of ethanol was refluxed for eight hours. The solvent was removed *in vacuo* and the residue was kept in a vacuum desiccator over phosphorus pentoxide for three days. The resulting mass was triturated with 15 ml of methanol, filtered, and recrystallized from methanol-ether. Yield 11.0 g ~ 56 %; m. p. 103—105° C. (Found: C 55.69; H 7.33; N 28.79. Calc. for $C_9H_{14}N_4O$: C 55.67; H 7.27; N 28.85).

b) *From cinnamic acid hydrazide (II)*. A mixture of 1.62 g (0.01 mole) cinnamic acid hydrazide (II), 1.5 ml (0.03 mole) of 100 % hydrazine hydrate and 4 ml of ethanol was refluxed for two hours. The solvent was removed *in vacuo* and the residual paste was kept in a vacuum desiccator over concentrated sulphuric acid over-night to remove excess hydrazine. The resulting solid mass was recrystallized from methanol-ether. Yield 1.05 g ~ 54 %; m. p. 101—103° C. The melting point was not depressed by admixture with the β-hydrazinodihydrocinnamic acid hydrazide from a) above.

Dibenzylidene-β-hydrazinodihydrocinnamic acid hydrazide (XII)

To a stirred solution of 194 mg (0.001 mole) of β-hydrazinodihydrocinnamic acid hydrazide in 5 ml of ethanol were added 5 ml of water, 0.3 ml of benzaldehyde and one drop of 4 N sulphuric acid. After 30 minutes the white precipitate was filtered off, washed with ethanol, and dried. Yield 340 mg ~ 92 %; m. p. 180—183° C. After recrystallization from methyl-cellosolve the melting point was 181—183° C. (Found: C 74.59; H 6.18; N 15.13. Calc. for $C_{23}H_{22}N_4O$: C 74.57; H 5.99; N 15.12).

Conversion of β-hydrazinodihydrocinnamic acid hydrazide (XI) to 3-phenyl-5-pyrazolidone (V)

3.88 g (0.02 mole) of β-hydrazinodihydrocinnamic acid hydrazide were distilled *in vacuo*. The fraction distilling at 195—200°/1.5 mm was collected. The distillate solidified to a white crystalline mass and was recrystallized from ethanol. Yield 1.67 g ~ 51 %; m. p. 101° alone and by admixture with 3-phenyl-5-pyrazolidone.

m-Nitrocinnamic acid hydrazide (XVI)

This compound was prepared from the potassium salt of *m*-nitrocinnamic acid in analogy with the preparation of cinnamic acid hydrazide (II). Yield of crude product 65 %. One recrystallization from ethanol yielded material melting at 200—201° C. (Curtius [2] reports 198°). The melting point was not depressed by admixture with *m*-nitrocinnamic acid hydrazide (m. p. 200—201°) prepared as described by Curtius [2].

3-(*m*-Nitrophenyl)-5-pyrazolidone (XIV) and the benzylidene compound of 3-(*m*-Nitrophenyl)-5-pyrazolidone (XIX)

These compounds were prepared as described by Curtius and Bleicher [3] for *m*-nitrocinnamic acid hydrazide (m. p. 139°) and benzylidene *m*-nitrocinnamic acid hydrazide (m. p. 206.5°), respectively. The yields and melting points were in agreement with the values reported by these authors.

β-Hydrazino-*m*-nitrodihydrocinnamic acid hydrazide (XX)

A mixture of 2.07 g (0.01 mole) of *m*-nitrocinnamic acid hydrazide (XVI), 1.5 ml (0.03 mole) of 100 % hydrazine hydrate and 4 ml of absolute ethanol was refluxed for 30 minutes. The ethanol was removed *in vacuo* and the residue was kept in a vacuum desiccator over concentrated sulphuric acid overnight. Crystallization from methanol-ether yielded 150 mg of a product, melting at 101—103°. (Found: C 45.04; H 5.50; N 29.28. Calc. for $C_9H_{12}N_6O_3$: C 45.19; H 5.48; N 29.28).

p-Nitrocinnamic acid hydrazide

This compound was prepared from the potassium salt of *p*-nitrocinnamic acid in analogy with the preparation of cinnamic acid hydrazide (II). Yield of crude product 70 %. After one recrystallization from ethanol the melting point was 215—216° C

(decomp.) alone and by admixture with *p*-nitrocinnamic acid hydrazide prepared as described by Gansser and Rumpf [4]. (Found: C 52.05; H 4.47; N 19.80. Calc. for $C_9H_9N_3O_3$: C 52.17; H 4.38; N 20.28).

p-Nitrocinnamoylazide

To a stirred solution of 300 mg of *p*-nitrocinnamic acid hydrazide in 5 ml of glacial acetic acid were added 200 mg of sodium nitrite. After 15 minutes at room temperature the yellow precipitate formed was filtered, washed with water, and recrystallized from chloroform-petrolether. M. p. 119° C (decomp.). (Found: C 49.65; H 2.81; N 25.75. Calc. for $C_9H_6N_4O_3$: C 49.53; H 2.77; N 25.67).

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X-Ray and Magnetic Study of the System Cobalt Selenium

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X-ray studies of cobalt selenides have revealed three intermediate solid phases.

The β-phase, probably Co_9Se_8, has a cubic face-centered structure with lattice constant $a = 10.431$ Å. The phase is stable at 400° C, but not at 600° C. Its structure is closely related to that of Co_9S_8 and pentlandite $(Fe,Ni)_9S_8$.

The γ-phase has a range of homogeneity between $CoSe_{1.02}$ and $CoSe_{1.37}$ in samples quenched from 600° C. In the range up to $CoSe_{1.15}$ the structure is hexagonal and of NiAs-like type, but from $CoSe_{1.20}$ on the structure is monoclinic. The lattice constants vary continuously between these limits:

CoSe	$a = 3.6294$ Å,	$c = 5.3006$ Å
$CoSe_{1.40}$	$a = 6.1372$ Å,	$c = 5.1976$ Å
	$b = 3.5673$ Å	$\beta = 91.22°$

Density determinations show that the solid solution within the γ-phase takes place by subtraction of cobalt atoms as the selenium content increases from CoSe on.

At 400° C the γ-phase is split into two phases, γ_1 with composition around $CoSe_{1.08}$ and NiAs-like structure and γ_2 with composition in the range $CoSe_{1.20}$ to $CoSe_{1.33}$ and monoclinic structure.

The δ-phase has a composition close to $CoSe_2$. It is confirmed that the structure is of the pyrite type with lattice constant varying from $a = 5.8611$ Å for $CoSe_{1.90}$ to $a = 5.8588$ Å for $CoSe_2$. A redetermination of the interatomic distances led to a selenium-selenium distance of 2.43 Å, which is shorter than hitherto supposed.

Magnetic measurements have been carried out by the Gouy method in the temperature range −183° to +450° C for samples in the range CoSe to $CoSe_2$. Except for a slight ferromagnetism in the CoSe range, the susceptibilities are rather low and do not follow Curie's law. Magnetic moments calculated by the Curie-Weiss law are 3.2 B.M. for $CoSe_{1.30}$ and 2.56 B.M. for $CoSe_2$ while the effective magnetic moments at 450° C are 1.7 B.M. and 2.0 B.M., respectively.

A compound of cobalt and selenium was obtained for the first time by Berzelius [1] by heating a mixture of the elements. Later on, CoSe was prepared by Little [2] and Fabre [3], who let selenium vapor react with cobalt in an atmosphere of hydrogen.

Fonzes-Diacon [4] prepared four more cobalt selenides. The Co_2Se_3 and $CoSe_2$ were formed by the action of hydrogen selenide on anhydrous cobaltous chloride, at and below dark red heat, respectively. If the hydrogen selenide was diluted with nitrogen and hydrogen chloride, Co_3Se_4 was obtained at dark red heat. In addition, Co_2Se was obtained as a silver-white substance if any of the other cobalt selenides were heated in hydrogen at bright red heat.

The existence of a cobalt selenide $3CoSe \cdot Co_2Se_3$, or Co_5Se_6, was claimed by Meyer and Bratke [5]. They heated a mixture of cobalt and selenium with potassium carbonate at 1 250° C and got a product containing 61.7 % Co and 38.0 % Se.

The compound CoSe has also been obtained by precipitation from an aqueous cobaltous salt solution by Reeb [6] and from an alcoholic solution by Moser and Atynski [7], who found the atomic ratio Co : Se = 1 : 1.032.

Of the six cobalt selenides (Co_2Se, CoSe, Co_5Se_6, Co_3Se_4, Co_2Se_3 and $CoSe_2$) described so far, only two have been verified by X-ray investigations. Goldschmidt [8] prepared CoSe by heating a mixture of the elements in hydrogen. The reaction product was examined with X-rays by Oftedal [9] who found that it had a structure of the *B*8-(NiAs-) type. The same conclusion regarding the structure of CoSe was reached by de Jong and Willems [10]. The lattice constants reported by Oftedal and de Jong and Willems are in good agreement.

Subsequently, de Jong and Willems [11] succeeded in preparing $CoSe_2$ by heating a mixture of CoSe and Se for two days at about 230° C. X-ray studies of the heterogeneous product showed the presence of a phase with cubic structure of the *C*2-(pyrite-) type. The lattice constant of $CoSe_2$ has been redetermined by Tengnér [12] and by Lewis and Elliott [13]. The latter authors also studied the interatomic distances in $CoSe_2$.

Cobalt monoselenide precipitates were studied by electron diffraction by Baroni [14] who recognized three different modifications. The amorphous α-CoSe was obtained by the action of ammonium selenide on a cobaltous salt solution. The β-CoSe with *B*8-type structure was obtained in the same way from a solution containing cobaltous acetate and acetic acid. The γ-CoSe, with a structure of the *B*13-(millerite-) type, was precipitated with hydrogen selenide from a cobaltous sulfate-sulfuric acid solution. The γ-CoSe was found to change very rapidly into β-CoSe.

The present study was undertaken as part of a series of investigations on the binary compounds of the iron group metals with sulfur, selenium and tellurium. It seemed especially interesting to determine the range of homogeneity of the CoSe phase, since a rather broad range might be expected, in analogy with the findings of Hägg and Kindström [15] for the FeSe phase. Magnetic measurements were also carried out to gain further insight in the interesting, but complicated magnetic behavior of these compounds.

EXPERIMENTAL

Metallic cobalt was prepared by igniting "Cobaltum carbonicum puriss" from Th. Schuchardt to oxide at 1 050° C and then reducing the oxide by means of purified hydrogen gas at 1 000° C. A spectrographic search for impurities revealed only the presence of nickel in an amount of less than 0.01 %. The analysis was kindly carried out by siv.

ing. S. Rutlin, Statens Råstofflaboratorium, Oslo. Some cobalt prepared from "Baker's Analyzed" Cobaltous Nitrate was also used.

Highly refined selenium was placed at our disposal by Bolidens Gruvaktiebolag, Sweden. It carried the following analysis: copper 0.0002 %, iron 0.0007 %, tellurium 0.003 %, sulfur 0.01 %, non volatile matter 0.003 %.

The alloys were synthesized by heating appropriate amounts of the elements in evacuated, sealed silica tubes at temperatures ranging between 500° and 1 070° C. Considerable devitrification of the silica tubes took place during a 24-hour heating period at high temperatures. Most of the alloys were therefore prepared at 600° C. The desired composition was in many cases obtained by adding selenium to Co_2Se or CoSe samples. The composition of the samples ranged between Co_2Se and $CoSe_2$ (33.33 and 66.67 atomic per cent selenium).

The light grey, sintered products were finely ground in an agate mortar and homogenized. One series of samples was kept at 600° C for 7 days and then quenched in ice water. Another series was kept at 400° C for 5 months and quenched from that temperature.

Some experiments were carried out to study the rate of formation of CoSe and $CoSe_2$. In one experiment, cobalt powder and selenium in atomic ratio 1 : 1 were heated at 350° C for 32 days. The X-ray photograph of the product showed the presence of large amounts of $CoSe_2$, indicating that equilibrium had not been attained. In another experiment, a mixture of CoSe and Se corresponding to the formula $CoSe_2$ was ground in the mortar and then heated at 225° C for 21 days. Only small amounts of $CoSe_2$ resulted from this treatment, and the heating was therefore continued at 300° C for 20 days. After this treatment more of the selenium had reacted with the CoSe phase to form $CoSe_2$, but unreacted selenium was still observed.

X-ray photographs were taken in 11.48 cm diameter powder cameras with asymmetric film mounting. Iron and copper radiation were used. The lattice constants are expressed in Ångström units on the basis of $\lambda FeK\alpha_1 = 1.93597$ Å and $\lambda CuK\alpha_1 = 1.54051$ Å. Lattice constant values from previous investigations, actually given in kX, are here expressed in Å by multiplication by the factor 1.00202. The mean error in the lattice constants is judged to be $\pm$ 0.01 % for the cubic structures, $\pm$ 0.02 % for the hexagonal structure and $\pm$ 0.03 % for the monoclinic structure.

RESULTS AND DISCUSSION

As a result of the phase analysis, the existence of three intermediate phases, β, γ and δ, was established.

1. The β-phase, Co_9Se_8 (?)

This phase was only present in the samples annealed at 400° C and has not been found by earlier investigators. It has a cubic structure with lattice constant:

$$a = 10.431 \text{ Å}.$$

No significant variation in the lattice constant with composition was observed and the homogeneity range of the β-phase is consequently supposed to be very narrow. As far as it could be judged from the powder photographs, the structure of the β-phase is face-centered and related to that found by Lindqvist, Lundqvist and Westgren [16] for Co_9S_8 and pentlandite $(Fe,Ni)_9S_8$.

The composition of the β-phase was found to be in the range of $CoSe_{0.80}$ or slightly below the Me_9X_8 composition. No indications of the phase Co_2Se claimed by Fonzes-Diacon [4] were found. A density determination carried out on $CoSe_{0.80}$ gave the result $d = 7.05$ g cm^{-3}, but the sample contained some cobalt and γ-phase as well. The density of the β-phase is therefore lower than

7.05 g cm^{-3} since the other phases present have higher densities. Assuming the formula Co_9Se_8 for the β-phase and four formula units in the unit cell the calculated density is 6.799 g cm^{-3}.

According to Lindqvist, Lundqvist and Westgren [16], the atomic arrangement in Co_9S_8 is as follows, in terms of the space group $Fm3m$ (O_h^5):

32 Co in (*f*) x, x, x; etc. 24 S in (*e*) y, 0, 0; etc.
4 Co in (*b*) ½, ½, ½; etc. 8 S in (*c*) ¼, ¼, ¼; etc.
with $x = 1/8$ and $y = 1/4$.

A calculation of the intensities of Co_9Se_8 was undertaken on the basis of these parameters, but the agreement between observed and calculated intensities was not very good. By increasing the y value to $y = 0.265$ a much better agreement was obtained. The relative intensities calculated using this value are listed in Table 1 together with those observed from photometer recordings on basis of $I_{obs}(440) = I_{calc}(440) = 100$.

The structure consists of a slightly distorted cubic close-packing of selenium atoms with cobalt atoms in the interstices. Eight ninths of the cobalt atoms are surrounded tetrahedrally by selenium, the rest are surrounded

Table 1. Observed and calculated intensities for the β-phase, assuming isomorphism with Co_9S_8, FeKα radiation.

$\sin^2\Theta \times 10^4$	*hkl*	I_{obs}	I_{calc}	$\sin^2\Theta \times 10^4$	*hkl*	I_{obs}	I_{calc}
261	111	8	6.6	5 769	733	6	5.1
335	200	5	2.9	—	{820	—	0.4
692	220	4	2.5		{644		0.2
940	311	53	41	—	{822	—	0.1
1 039	222	85	78		{660		0.3
1 382	400	8	4.4	6 458	{751	14	5.1
1 641	331	18	13		{555		3.4
1 727	420	2	0.9	6 545	662	25	22
2 075	422	2	0.9	6 889	840	5	1.6
2 332	{511 {333	36	{23 {0.1	7 149	{911 {753	5	0.1 3.5
2 761	440	*100*	*100*	—	842	—	0.1
3 014	531	1	0.6	7 579	664	1	0.6
3 115	{600 {442	1	{0.2 {0.2	7 839	931	15	12
				8 267	844	76	83
3 438	620	1	0.9		{933		0.5
3 706	533	5	4.2	8 526	{771	8	5.9
3 786	622	43	26		{755		0.3
4 141	444	2	0.9	—	{10,0,0	—	0.3
4 387	{711 {551	6	{2.2 {0.2		{860		0.2
4 477	640	1	0.4	8 954	{10,2,0	3	1.3
4 826	642	1	0.8		{862		0.5
5 070	{731 {553	13		9 212	{951 {773	37	{38 {0.0
5 510	800	24	18	9 300	{10,2,2 {666	37	{28 {9.4

octahedrally. The octahedral Co-Se distances are 2.46 Å while the tetrahedral Co-Se distances range between 2.26 and 2.35 Å.

2. *The γ-phase, CoSe — Co_3Se_4*

The cobalt-rich limit of the homogeneity range of the γ-phase is markedly different in the 600° and the 400° C series. X-ray powder photographs of these series show that CoSe is a two-phase preparation at 400° C, containing about equal amounts of the β- and γ-phases, while it only contains the γ-phase at 600°. The structure of the γ-phase is of the *B*8-(NiAs-) type and the lattice constants vary considerably with temperature and composition.

The results of the calculations for the 600° C samples are given in Fig. 1 and in Table 2. It appears from the figure that even at this temperature the γ-phase does not have the exact composition CoSe when it exists in equilibrium with the α-phase (cobalt). The cobalt-rich composition limit for the γ-phase is probably close to $CoSe_{1.02}$ under the given conditions.

Table 2. Lattice constants of the γ-phase, 600° C series.

Composition	a, $(b\sqrt{3})$ in Å	b in Å	c in Å	β
$CoSe_{0.80}$	(6.2841)	3.6281	5.3008	(90°)
$CoSe_{1.00}$	(6.2864)	3.6294	5.3006	(90°)
$CoSe_{1.05}$	(6.2745)	3.6226	5.2954	(90°)
$CoSe_{1.10}$	(6.2545)	3.6111	5.2850	(90°)
$CoSe_{1.15}$	(6.2297)	3.5967	5.2713	(90°)
$CoSe_{1.20}$	6.2086	3.5846	5.2661	90.5°
$CoSe_{1.25}$	6.1863	3.5805	5.2503	90.90°
$CoSe_{1.30}$	6.1475	3.5766	5.2161	91.45°
$CoSe_{1.35}$	6.1373	3.5695	5.2056	91.47°
$CoSe_{1.40}$	6.1372	3.5673	5.1976	91.22°

The lattice constants found by Oftedal [9] for CoSe * ($a = 3.621 \pm 0.003$ Å, $c = 5.289 \pm 0.004$ Å) and those found by de Jong and Willems [10] ($a = 3.61$ Å, $c = 5.28$ Å) agree fairly well with those reported here. The values determined by Baroni [14] ($a = 3.51$ Å, $c = 5.12$ Å) by means of electron diffraction are considerably lower.

With increasing selenium content the lattice constants of the γ-phase are found to decrease almost linearly to the composition $CoSe_{1.15}$. At the composition $CoSe_{1.20}$ (54.55 atomic per cent Se) a lowering of the symmetry from hexagonal to monoclinic is observed. This change in symmetry is accompanied by a sharp bend in the curve for the *b*-axis. The angular deformation increases from $\beta = 90.5°$ at $CoSe_{1.20}$ to $\beta = 91.47°$ at $CoSe_{1.35}$, and then decreases slightly until the composition limit of the γ-phase is reached. In the monoclinic range the *c*-axis decreases more rapidly with composition than it does in the hexago-

* The preliminary values listed by Goldschmidt [8] ($a = 3.594 \pm 0.003$ Å, $c = 5.277 \pm 0.005$ Å) are somewhat lower than those later reported by Oftedal [9].

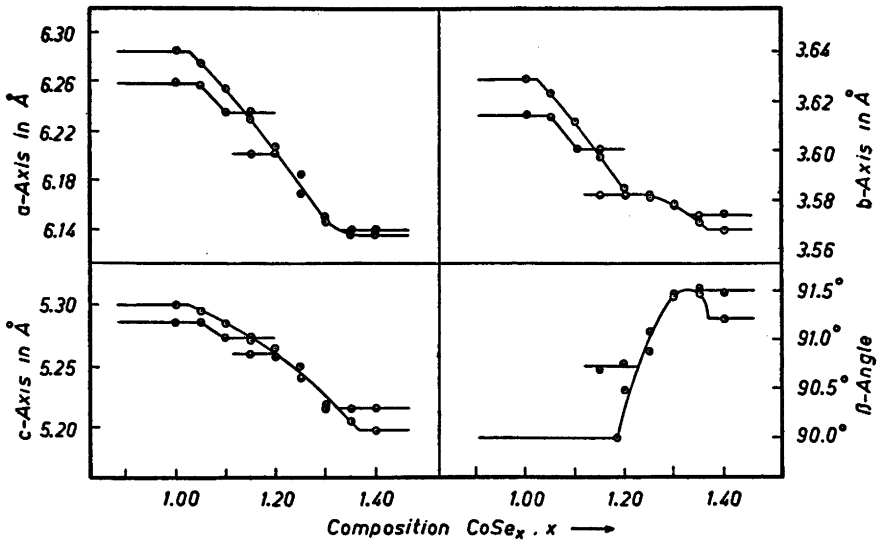

Fig. 1. Lattice constants of the γ-phase, CoSe-Co_3Se_4, after quenching from 600°C ⊙ and 400°C ◒.

nal range, while the opposite is true for the *b*-axis. The decrease in the *a*-axis is almost linear within the whole homogeneity range.

As a result of the lattice constant determinations, the selenium-rich limit for the γ-phase in the 600° C series is found close to the composition $CoSe_{1.37}$ (57.81 atomic per cent Se). On the X-ray photographs of $CoSe_{1.40}$ lines from a new phase, the δ-phase ($CoSe_2$), are found. The compounds Co_3Se_4 found by Fonzes-Diacon [4] and Co_5Se_6 by Meyer and Bratke [5] both fall within the homogeneity range of the γ phase and need not be considered as separate phases. Neither the existence of the compound Co_2Se_3, claimed by Fonzes-Diacon [4], nor the polymorphism of CoSe observed by Baroni [14] has been confirmed by the present authors.

The variation in density with composition for the γ-phase has been studied in some detail. The densities were determined at 25° C by the vacuum pycnometric method with kerosene as displacement liquid. There is an appreciable

Table 3. Observed and calculated densities in g cm^{-3} of the γ-phase.

Sample	d_{obs}	d_{calc}	Sample	d_{obs}	d_{calc}
$CoSe_{1.00}$	7.377	7.572	$CoSe_{1.20}$	7.232	7.257
$CoSe_{1.05}$	7.377	7.453	$CoSe_{1.25}$	7.196	7.200
$CoSe_{1.10}$	7.304	7.375	$CoSe_{1.30}$	7.135	7.183
$CoSe_{1.15}$	7.244	7.323	$CoSe_{1.35}$	7.071	7.142
			$CoSe_{1.40}$	7.072	—

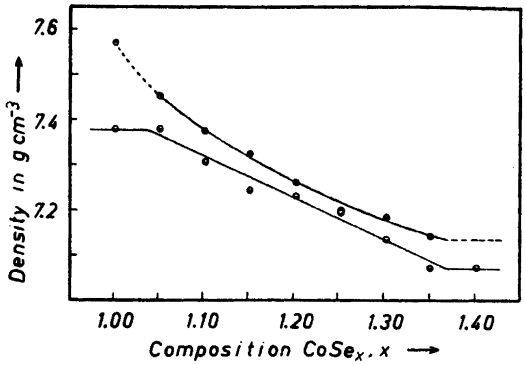

Fig. 2. Observed density ◒ and calculated density ◓ of the γ-phase.

decrease in density with increasing selenium content, indicating that the solid solution is of the subtractive type. The observed density values and those calculated on the basis of subtraction of cobalt atoms from the CoSe structure when the selenium content increases are listed in Table 3.

From Fig. 2 it can be seen that the agreement between observed and calculated densities is very satisfactory in the region $CoSe_{1.05}$ to $CoSe_{1.35}$ and the hypothesis of a subtractive solid solution is therefore fully confirmed in this region. A discrepancy is, however, present for $CoSe_{1.00}$, but there is an obvious reason for this and that is the instability of $CoSe_{1.00}$ with regard to the β-phase and the selenium richer γ-phase. Since both these phases have lower densities than stoichiometric CoSe, a partial decomposition of CoSe on quenching will lead to lower density values.* Lines from the β-phase are not observed on the X-ray photographs of the quenched CoSe, but this may be due to the fine state of division of the β-phase in its early stage of formation.

Table 4. Lattice constants of the γ_1- and γ_2-phases, 400° C series.

Composition	a, ($b\sqrt{3}$) in Å	b in Å	c in Å	β
$CoSe_{1.00}$	(6.2597)	3.6141	5.2863	(90°)
$CoSe_{1.05}$	(6.2573)	3.6128	5.2861	(90°)
$CoSe_{1.10}$	(6.2366)	3.6006	5.2730	(90°)
$CoSe_{1.15}$	(6.237)	3.600	5.273	(90°)
	6.200	3.580	5.256	90.7°
$CoSe_{1.20}$	6.2024	3.5813	2 × 5.2575	90.76°
$CoSe_{1.25}$	6.1692	3.5809	2 × 5.2400	91.09°
$CoSe_{1.30}$	6.1499	3.5773	2 × 5.2178	91.47°
$CoSe_{1.35}$	6.1393	3.5732	2 × 5.2149	91.56°
$CoSe_{1.40}$	6.1390	3.5736	2 × 5.2148	91.48°

* Experiments carried out by cand.real. J. Vihovde in this institute gave $d = 7.333$ g cm^{-3} for a CoSe sample that had been quenched from 800° C and $d = 7.165$ g cm^{-3} as mean value for four samples that had been cooled in the furnace.

The results of the lattice constant determinations for the samples annealed at 400° C are found in Table 4 and Fig. 1. In these samples the β- and γ-phases are coexistent up to the composition $CoSe_{1.05}$. At the composition $CoSe_{1.10}$ only the γ-phase is observed on the X-ray photographs. When the composition is increased to $CoSe_{1.15}$ two phases are present, while the preparations $CoSe_{1.20}$ to $CoSe_{1.35}$ contain only one phase, with lattice constants which seem to vary continuously with the composition.

The γ-phase, which exists in the range $CoSe_{1.02}$ to $CoSe_{1.37}$ in the 600° C series, is thus split into two phases, γ_1 and γ_2 at 400° C. The γ_1-phase is hexagonal and has a range of homogeneity around $CoSe_{1.08}$, while the monoclinic γ_2-phase has a range of homogeneity from $CoSe_{1.20}$ to $CoSe_{1.33}$. Weak additional lines in the monoclinic range require a doubling of the c-axis for their explanation, and this is indicated in the table.

According to the present study, the homogeneity range of the γ-phase is rather susceptible to temperature changes. This reflects important variations in the entropy of the cobalt selenides with temperature and composition. It is also seen how the phases tend to accept more definite compositions as the temperature is lowered. Analogous observations have already been made by Grønvold and Haraldsen [17] for the $B8$-like phase in the system iron-sulfur and iron-selenium.

In the hexagonal γ-phase, each cobalt atom is surrounded by six selenium atoms at the corners of a right trigonal prism. The cobalt-selenium distances are 2.48 Å for CoSe and 2.46 Å for $CoSe_{1.15}$. These distances are much shorter than the sum of the ionic radii, but they compare very favorably with the sum of the metallic radii as given by Pauling [18]. If the radii of cobalt and selenium are corrected for eight and six coordination, respectively, while the normal valencies are maintained, the radius sum is 1.20 Å + 1.31 Å = 2.51 Å. This is only 0.03 Å higher than experimentally found for CoSe.

Each cobalt is further surrounded by two cobalt atoms at the distance $c/2$. This distance is rather short in the γ-phase, 2.65 Å for CoSe and 2.64 Å for $CoSe_{1.15}$, so that exchange interactions between the paramagnetic ions (leading to antiferromagnetism and ferromagnetism) might be expected.

The selenium atoms form an approximately hexagonal close-packing, the shortest distance (3.38 Å for CoSe) between atoms in adjacent layers being smaller than between atoms in the layer (3.63 Å).

3. The δ-phase, $CoSe_2$

At the composition $CoSe_{1.90}$, the X-ray photographs show the δ-phase as major constituent, but a few lines from the γ-phase are still visible. At the composition $CoSe_{2.00}$ all lines from the γ-phase have disappeared and a slight shift in the positions of the back reflection lines of the δ-phase is noted. Thus, the cobalt-rich limit of the homogeneity range of the δ-phase lies slightly below the composition $CoSe_2$.

In agreement with earlier observations, the structure of $CoSe_2$ is cubic of the $C2$- (pyrite-) type. The lattice constants are listed in Table 5. Tengnér's [12] result and that of Lewis and Elliott [13] are in good accord with ours for $CoSe_{2.00}$, while that of de Jong and Willems [11] is closer to our value for $CoSe_{1.90}$. The

Table 5. Lattice constants of the δ-phase, $CoSe_2$.

Composition	a in Å	Authors
$CoSe_{1.90}$	5.8611	Present
$CoSe_{2.00}$	5.8588	Present
Unknown	5.866 ± 0.003	de Jong and Willems [11]
$CoSe_2$	5.857	Tengnér [12]
$CoSe_2$	5.857 ± 0.005	Lewis and Elliott [13]

observed density of $CoSe_{2.00}$ is 7.087 g cm^{-3} at 25° C, which compares favorably with the X-ray density 7.160 g cm^{-3}.

A redetermination of the parameter x was undertaken in order to establish the interatomic distances more accurately. Small changes in this parameter have a marked influence on the selenium-selenium distance, but rather little on the cobalt-selenium distance. The best agreement between observed and calculated intensities was obtained with $x = 0.380$. In Table 6 are listed

Table 6. Observed and calculated intensities for $CoSe_2$ with $x = 0.380$, Cu radiation.

$\sin^2\Theta \times 10^4$	*hkl*	I_{obs}	I_{calc}	$\sin^2\Theta \times 10^4$	*hkl*	I_{obs}	I_{calc}
—	111	—	0.0	6 055	531	1	0.0
692	200	18	30	6 229	600	5	0.2
868	210	55	100		442		4.0
1 042	211	54	86	6 405	610	7	4.8
1 386	220	12	20	6 572	611	20	5.2
1 556	221	1	0.3		532		11
1 908	311	48	66	6 921	620	2	2.8
2 082	222	4	5.2		621		0.4
2 243	320	26	28	—	540	—	0.1
2 420	321	*40*	*40*		443		0.2
2 769	400	8	5.6	—	541	—	0.2
	410		0.2	7 439	533	14	11
—	322	—	0.1	7 613	622	3	3.2
—	411	—	0.2	7 777	630	19	6.8
—	331	—	0.4		542		7.9
3 457	420	5	5.9	7 956	631	16	13
3 628	421	24	18	8 301	444	5	2.6
3 797	332	11	8.6	—	632	—	0.7
4 147	422	5	4.5	—	543	—	0.2
—	430	—	0.1	8 817	711	2	0.2
—	431	—	0.3		551		0.4
4 653	511	24	18	8 988	640	9	11
	333		3.6	9 160	720	30	14
5 005	520	24	4.4		641		17
	432		15		721		23
5 179	521	17	12	9 334	633	40	11
5 538	440	34	21		552		10
—	522	—	0.0	9 677	642	12	18
	441		0.2	—	722	—	0.3
—	433	—	0.1		544		0.6

the results of the intensity measurements and calculations, together with the observed $\sin^2\Theta$ values of the α_1 reflections. The observed intensities are from photometric recordings of the film and the observed and calculated intensity of the 321 reflection have been set equal.

In the $CoSe_2$ structure, each cobalt atom is surrounded octahedrally by six selenium atoms at the distance 2.42 Å. Each selenium atom is surrounded tetrahedrally by three cobalt atoms at the same distance and by one selenium atom at the distance 2.43 Å. These distances are explained satisfactorily by means of Pauling's [18] metallic radii. With the coordination number six for cobalt and four for selenium the radius sum is calculated to be 1.16 Å + 1.26 Å = 2.42 Å, in perfect agreement with the observed value. The calculated selenium radius 1.26 Å is, however, somewhat high in comparison with the observed value 1.22 Å.

According to Pauling and Huggins [19], the tetrahedral covalent radius of selenium is 1.14 Å, and a selenium-selenium distance of 2.28 Å is therefore expected if the chemical bonds in the $CoSe_2$ structure were as proposed by these authors for pyrite. Their suggestion that the distance Se-Se = 2.54 Å found by de Jong and Willems [11] was too large has been confirmed by Lewis and Elliott [13], who found Se-Se = 2.49 ± 0.04 Å. Neither that value nor the one found by us (2.43 Å) is, however, equal to the tetrahedral covalent selenium distance (2.28 Å).

4. Magnetic properties of the cobalt selenides

Samples in the range CoSe to $CoSe_2$ were studied magnetically by the Gouy method at temperatures ranging from —183° to +450° C. The results of the measurements are found in Table 7. Field strength dependence of the susceptibility was noted in the range CoSe to $CoSe_{1.20}$. For CoSe the effect was rather large, but this is not surprising since the X-ray results indicated that CoSe was a two phase preparation in the 600° C series (Co + $CoSe_{1.02}$). The ferromagnetism in CoSe disappeared at about 770° C, or far below the ferromagnetic Curie temperature of cobalt, which is 1121° C according to Myers and Sucksmith [20]. If, therefore, the ferromagnetism is due to cobalt, its disappearance might be interpreted to be the result of increased solubility of cobalt in the γ-phase at higher temperatures. The cobalt-rich limit for the γ-phase should thus be $CoSe_{1.00}$ at about 770° C.

While the presence of metallic cobalt is probably the cause of the high magnetism of CoSe, the weak field strength dependence of the selenium-richer samples seems to be a property of the hexagonal γ-phase. Such a field strength dependent susceptibility has often been observed (see for example the results found by Haraldsen, Rosenqvist and Grønvold [21] for the chromium antimonides), and it may be due to what Néel [22] terms ferrimagnetism.

One can expect ferrimagnetism for substances where antiferromagnetic interactions are present and the sublattices are unequally populated with magnetic ions (*e.g.* because of non-stoichiometric composition of the phase in question). An unbalanced antiferromagnetism may very well be present in structures of *B*8-like type where metal ions are subtracted with increasing metalloid content, and this is a possible explanation for the field strength dependent susceptibility observed for samples in the region $CoSe_{1.05}$ to $CoSe_{1.20}$.

Table 7. Magnetic susceptibility of the cobalt selenides, $\chi_g \times 10^6$.

Composition	Temperature in °C					
	− 183	−78	+20	+150	+ 300	+450
$CoSe_{1.00}$ *	810	810	812	932	1 049	1 021
$CoSe_{1.05}$ *	66	52	52	56	56	56
$CoSe_{1.10}$ *	28	29	30	33	35	34
$CoSe_{1.15}$ *	9.7	9.3	9.3	9.4	9.4	9.4
$CoSe_{1.20}$ *	5.6	5.5	5.2	5.6	5.5	5.5
$CoSe_{1.25}$	3.88	3.53	3.33	3.10	2.99	2.97
$CoSe_{1.30}$	3.96	3.86	3.54	3.25	3.05	2.87
$CoSe_{1.35}$	4.11	3.59	3.21	2.82	2.66	2.50
$CoSe_{1.40}$	4.06	3.60	3.18	2.72	2.51	2.46
$CoSe_{1.50}$	4.72	4.04	3.55	3.08	2.78	2.54
$CoSe_{1.70}$	5.93	4.63	4.00	3.38	2.85	2.60
$CoSe_{1.80}$	6.21	4.95	4.21	3.62	3.15	2.69
$CoSe_{1.90}$	6.83	5.36	4.66	3.98	3.23	2.73
$CoSe_{2.00}$	6.73	5.42	4.60	4.01	3.33	2.68

* These samples are ferromagnetic and the values refer to H_{max} = 4 117 Ørsted.

The samples richer in selenium than $CoSe_{1.20}$ are completely paramagnetic at all temperatures used in this work. In Fig. 3 are found the molar susceptibilities of the paramagnetic samples as functions of the composition. The susceptibility varies in a non-linear way within the monoclinic range of the γ-phase and the selenium-rich composition limit of the γ-phase is seen to be close to $CoSe_{1.37}$ in perfect agreement with the X-ray result. The molar susceptibilities have been corrected for the diamagnetism of the cobalt and selenium ions with the values $\chi_{Co++} = -18.5 \times 10^{-6}$ per mole according to Klemm [23] and $\chi_{Se--} = -47.58 \times 10^{-6}$ per mole according to Angus [24].

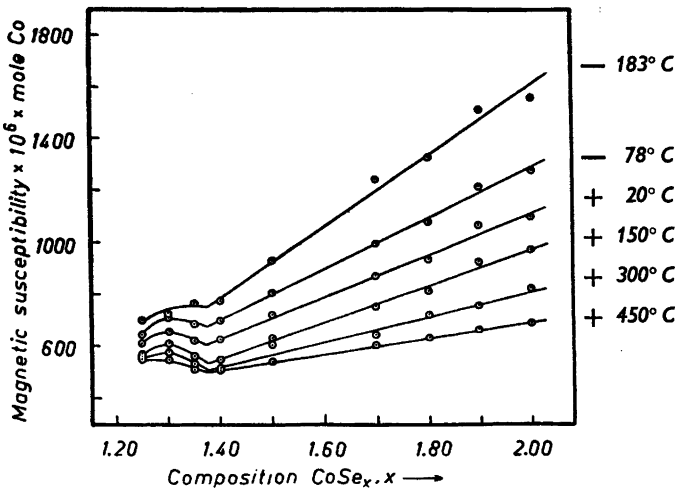

Fig. 3. Paramagnetic molar susceptibility of cobalt selenides in the range $CoSe_{1.25}$ to $CoSe_{2.00}$ after correction for induced diamagnetism.

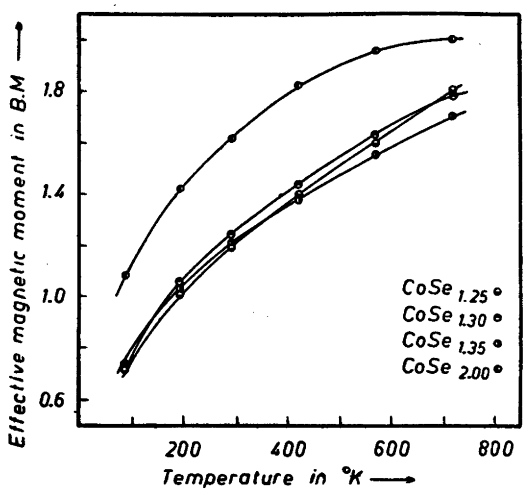

Fig. 4. Effective magnetic moments of the γ- and δ-phases.

A strictly linear relationship between $1/\chi_{mole}$ and T does not exist for the monoclinic γ-phase, but the approximate linearity can be described with a Weiss constant $\Delta \approx 2\,100$°K for $CoSe_{1.25}$, which leads to a magnetic moment $\mu = 3.4$ B.M. The corresponding values for $CoSe_{1.30}$ are: $\Delta \approx 1\,700$°K and $\mu = 3.2$ B.M. and for $CoSe_{1.35}$: $\Delta \approx 1\,100$°K and $\mu = 2.7$ B.M. The physical significance of magnetic moments obtained by the Curie-Weiss law ($\mu = 2.828 \sqrt{\chi_{mole}(T + \Delta)}$ Bohr magnetons) with large Δ values is rather dubious, especially in cases like the present one, where the law fails to be valid in the high temperature region.

In such cases it is presumably more satisfactory to calculate the effective magnetic moment ($\mu_{eff} = 2.828 \sqrt{\chi_{mole} T}$ B.M.) and study its variation with temperature. This has been done for the samples $CoSe_{1.25}$, $CoSe_{1.30}$ and $CoSe_{1.35}$ and the results are given in Fig. 4. The effective magnetic moment shows the same temperature dependence for all three samples and raises from about 0.7 B.M. at 90°K to 1.7 B.M. at 723°K. The values level off to some extent above room temperature, but they do not become constant.

In magnetic respect the behavior of the δ-phase, $CoSe_2$, is not much different from that of the monoclinic γ-phase. The magnetic susceptibility is wholly independent of the field strength and the Curie-Weiss law is fulfilled with $\Delta = 440$°K. This gives a magnetic moment for the δ-phase of $\mu = 2.56$ B.M. From Fig. 4 it can be seen how the effective magnetic moment of $CoSe_2$ varies with temperature and reaches the value 2.0 B.M. at 723°K.

According to the present results, the magnetic moments of the paramagnetic cobalt selenides are much lower than usually observed for ionic cobaltous compounds. In such cases a "spin only" value of 3.87 B.M. is expected, while the actual values lie in the range 4.3 to 5.6 B.M. (see Figgis and Nyholm [25]). Low magnetic moments (1.8 to 2.9 B.M.) are, however, observed for octahedral covalent complexes of divalent cobalt and a magnetic moment of 1.73

B.M. is expected both from the crystalline field theory according to Van Vleck [26] and from the valence bond theory by Pauling [27]. This is in reasonable agreement with the effective magnetic moment found both for the γ- and the δ-phase above room temperature. Another possible way of explaining low magnetic moments is that there are interactions between the paramagnetic neighbors leading to antiferromagnetism. Such interactions are presumably more probable in the γ-phase where the nearest cobalt atoms are only 2.65 Å apart than in the δ-phase where they are 4.14 Å apart.

According to the valence bond theory the magnetic moment of the δ-phase is not expected to rise above 1.73 B.M. unless there is an orbital contribution to the magnetic moment of the unpaired electron. The magnetic moment found for $CoSe_2$ might thus be taken as an indication that orbital contributions are present and that the unpaired electron is in a 3d orbital instead of 5s as suggested by Figgis and Nyholm [25] for octahedral covalent compounds of divalent cobalt. The d^2sp^3 bonds should then make use of one 3d and one 4d orbital of cobalt. A decrease in magnetic moment at low temperature is understandable in this picture as a result of increased 3d character of the d^2sp^3 bonds and interaction between the cobalt atoms.

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Investigations of the Properties and Mechanism of the Uridine Diphosphate Glucose Pyrophosphorylase Reaction

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A purification procedure for the uridine diphosphate glucose pyrophosphorylase is described, and the purified enzyme preparation is used to determine equilibrium, pH dependence, Mg^{++} requirement, and Michaelis constant for the UDPG pyrophosphorylase reaction.

Isotopic exchange experiments show that the purified enzyme catalyzes an incorporation of ^{32}P-labelled pyrophosphate into uridine triphosphate in the absence of glucose-1-phosphate. Likewise ^{32}P-labelled glucose-1-phosphate is incorporated into uridine diphosphate glucose in the absence of pyrophosphate, while ^{14}C uridine monophosphate is not incorporated into either of the substrates. These findings are discussed in relation to the mechanism of the reaction.

In 1950 Leloir and coworkers [1] isolated from yeast a nucleotide, uridine diphosphate glucose (UDPG) *, consisting of a nucleoside, linked to glucose by a pyrophosphate bridge. Shortly afterwards it was shown by Kalckar and Cutolo [2] that yeast contained an enzyme, which brought about a pyrophosphorolytic cleavage of UDPG with simultaneous formation of UTP and G-1-P:

$$\text{UDPG} + \text{PP} \rightleftharpoons \text{UTP} + \text{G-1-P} \qquad (1)$$

Pyrophosphorolytic cleavage of a nucleoside diphosphate compound was first demonstrated by Kornberg [3,4], who isolated from yeast and liver an enzyme which reversibly catalyzed the condensation of ATP with nicotinamide mononucleotide to split out PP and form the dinucleotide diphosphopyridine nucleotide. Later, Schrecker and Kornberg [5] demonstrated an analogous reaction with another dinucleotide, flavine adenine dinucleotide.

Reactions of this kind have subsequently been shown to be a more generally operating mechanism in group transfer reactions. Thus the biosynthesis of

* The following abbreviations are used: UTP for uridine triphosphate, G-1-P for glucose-1-phosphate, Gal-1-P for galactose-1-phosphate, G-6-P for glucose-6-phosphate, PP for inorganic pyrophosphate, ATP for adenosine triphosphate, ADP for adenosine diphosphate, UDP for uridine diphosphate, UMP for uridine monophosphate, UDPAG for uridine diphosphate N-acetyl glucosamine, TPN for triphosphopyridine nucleotide, GDPM for guanosine diphosphate mannose and Tris for tris(hydroxymethyl) aminomethane.

UDPAG and of GDPM in both cases involve a nucleoside triphosphate and the corresponding sugar phosphate ester [6,7].

Moreover, Kennedy and Weiss [8] have recently shown that in the biosynthesis of lecithin in liver a pyrophosphorylase is involved which forms cytidine diphosphate choline and pyrophosphate from cytidine triphosphate and choline phosphate. Kalckar [9] has proposed the name nucleotidyl transferases for this class of enzymes. However, for the individual enzymes of the class the term 'pyrophosphorylase' appears to be most descriptive.

The UDPG pyrophosphorylase which is abundantly present in yeast has also been shown to occur in liver [10], in muscle and brain tissue [11] and in leaves of spinach [12]. In this report a study of the purification and properties of the yeast enzyme and of the kinetics and mechanism of the reaction will be presented.

METHODS

Materials

UDPG was prepared as described by Leloir *et al.*[1] The preparation used throughout was a solution of about 60 % purity (as estimated by enzymatic analysis with the purified UDPG pyrophosphorylase and by chromatographic methods), and contained besides the UDPG some UDPAG together with small amounts of UDP and UMP.

$^{32}P^{32}P$ was obtained according to Kornberg and Pricer [13] by dehydration of ^{32}P-labelled Na_2HPO_4 and subsequent separation on a Dowex 1 Cl^- column.

^{32}P-labelled G-1-P was prepared by the method of McCready and Hassid [14], applying ^{32}P-labelled Na_2HPO_4 in the phosphorylase reaction.

ATP, ADP, UMP, and TPN were commercial products.

^{14}C-labelled UMP was prepared by incubating 2-^{14}C-labelled orotic acid with liver homogenate [15]. The sample applied in the exchange experiments was generously donated by Dr. Robert Hurlbert. The activity was 32 000 cts/min./μmole.

The *Saccharomyces fragilis* preparation was a freeze-dried product, grown on a galactose-containing medium *.

Enzymes

Glucose-6-phosphate dehydrogenase (Zwischenferment) was prepared by the method of LePage and Mueller [16]. The preparation obtained in this way was free of 6-phosphogluconic acid dehydrogenase, but contained large amounts of UDPG pyrophosphorylase. A glucose-6-phosphate dehydrogenase free of UDPG pyrophosphorylase was obtained in the following way: 100 mg of the G-6-P dehydrogenase was dissolved in 1 ml Tris buffer. The mixture was spun, and to the clear supernatant (0.9 ml) was added 1.35 ml saturated ammonium sulphate. After 20 min. at 0° C the precipitate was removed by centrifugation. To the supernatant (2.1 ml) was added 1.75 ml saturated ammonium sulphate, and after 20 min. at 0° C the precipitate was collected by centrifugation and dissolved in 0.2 ml Tris buffer. This solution was highly active with respect to glucose-6-phosphate dehydrogenase and usually devoid of UDPG pyrophosphorylase. Traces of the latter enzyme could be removed by dialysis against cold distilled water. The enzyme was stable for several weeks when kept at −20° C.

Nucleoside diphosphokinase was not prepared as such, but when the action of this enzyme was wanted, advantage was made of its abundant presence in the G-6-P dehydrogenase preparation.

Phosphoglucomutase was prepared according to Najjar [17]. In most experiments the "second heat filtrate" was used. This preparation contained sufficient glucose diphosphate for assay of G-1-P, so that extra addition of coenzyme was unnecessary.

Hexokinase was a commercial product obtained from Pabst Breweries, Inc.

* My thanks are due to Civilingeniør B. Steinhardt, Novo Terapeutisk Laboratorium, Copenhagen, for growing and harvesting a larger amount of this microorganism.

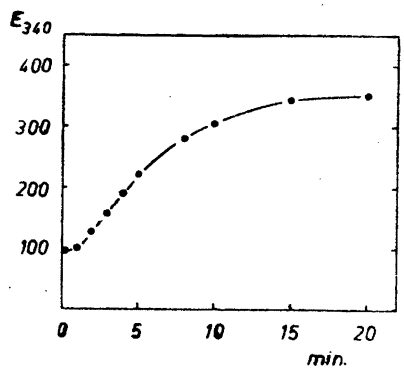

Fig. 1. Spectrophotometric demonstration of the UDPG pyrophosphorylase reaction.

Reaction mixture: 0.8 ml Tris (0.05 M, pH 7.2, 0.005 M $MgCl_2$), 25 µl phosphoglucomutase, 25 µl cysteine (10 mg/ml), 10 µl G-6-P dehydrogenase solution, containing also UDPG pyrophosphorylase, and 10 µl PP (0.1 M). Time zero indicates addition of PP. First reading was taken at 15 seconds.

Assay of UDPG pyrophosphorylase activity

A unit of enzyme activity was defined as the amount causing the splitting of 0.1 µmole/min. Specific activity is defined as units per mg of protein. A rise in extinction of 0.622 per 0.1 µmole at 340 mµ was used as basis for the calculations [18].

Protein determinations in the enzyme preparations were carried out according to the method of Lowry *et al.*[19] or, in the crude extracts, according to Bücher [20].

The usually applied and most convenient assay was a spectrophotometric determination, consisting of a sequence of enzymatic processes. Care therefore had to be taken that the UDPG pyrophosphorylase was the velocity limiting factor. The following sequence of reactions took place:

UDPG + PP → UTP + G-1-P
G-1-P → G-6-P
G-6-P + TPN+ + H_2O → 6-phosphogluconate + TPNH + H^+

The G-1-P formed by the pyrophosphorylase reaction was converted to G-6-P by addition of mutase, and G-6-P was oxidized by addition of TPN and glucose-6-phosphate dehydrogenase [21]. The formation of reduced TPN was finally measured by following the rise in extinction at 340 mµ. Excess of mutase and glucose-6-phosphate dehydrogenase was secured by regular checking in separate controls.

The assay was carried out in 1 ml quartz absorption cells, and the components of the assay system were as follows: 800 µl Tris (0.05 M, pH 7.2, 0.005 M $MgCl_2$), 25 µl mutase (3 mg/ml), 25 µl cysteine (10 mg/ml), 10 µl glucose-6-phosphate dehydrogenase (free of UDPG pyrophosphorylase), 50 µl UDPG solution (60 % purity, 7 µmole UDPG/ml), 20 µl TPN (0.01 M), and 10–20 µl of the UDPG pyrophosphorylase solution. Initial readings were taken at 340 mµ (the UDPG preparation contained minute amounts of G-1-P, which usually caused a rise in extinction of about 0.010–0.020, when the G-6-P dehydrogenase was added). When readings were constant, 10 µl 0.1 M PP was added to start the pyrophosphorylase reaction, and readings were taken every 30 seconds. Final and stable extinctions were usually obtained after 25–30 minutes.

The reaction, when demonstrated spectrophotometrically, shows a pronounced lag-period (see Fig. 1), which will be discussed later. This lag-period does not, however, influence the quantitative aspect of the assay, and the maximum rate (optical density change at 340 mµ) obtained after the lag-period was proportional to the amount of enzyme added.

Occasionally the pyrophosphorylase activity was measured by means of the nucleoside diphosphokinase-hexokinase system as described by Berg and Joklik [22]. The sequence of reactions was as follows:

$$\begin{aligned} &\text{UDPG} + \text{PP} \rightarrow \text{UTP} + \text{G-1-P} \\ &\text{UTP} + \text{ADP} \rightarrow \text{UDP} + \text{ATP} \\ &\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{G-6-P} \\ &\text{G-6-P} + \text{TPN}^+ + \text{H}_2\text{O} \rightarrow \text{6-phosphogluconic acid} + \text{TPNH} + \text{H}^+ \end{aligned}$$

It is seen that in this case it is the UTP, formed in reaction (1) which is measured. The assay system contained: 900 μl Tris (0.05 M, pH 7.2, 0.005 M $MgCl_2$), 10 μl G-6-P dehydrogenase (which also contained the nucleoside diphosphokinase), 20 μl ADP, 0.002 M, 25 μl hexokinase (3 mg protein per ml), 1.0 μl glucose, 0.01 M, 20 μl TPN (0.01 M), 10–20 μl UDPG pyrophosphorylase and 20 μl UDPG, 0.007 M. 10 μl PP, 0.1 M, started the reaction which likewise was followed at 340 mμ. The G-6-P dehydrogenase as well as the hexokinase were checked for absence of phosphoglucomutase. The assay here described may be used for demonstrating pyrophosphorolysis of UDP glycosyl compounds other than UDPG.

All enzymatic reactions were, unless otherwise stated, carried out at room temperature. In large scale experiments, when the reaction products, primarily UTP, were isolated, the reaction was stopped by adding $HClO_4$ to a final concentration of 2 %. Proteins were removed by spinning, and the nucleotides were adsorbed on a suitable amount of norite and subsequently eluted with 50 % ethanol.

Before use the norite was washed by shaking for 2 hours with 100 volumes of 2 % hexanol [23]. The suspension was filtered and washed thoroughly with distilled water, followed by drying in the air. Systematic experiments with a solution of known nucleotide content were carried out in order to fix the optimal conditions for adsorption and elution; as a result of these experiments the following standard procedure was applied throughout: To the perchloric acid solution was added 5 mg norite per 0.1 μmole nucleotide. After 2 minutes the mixture was spun; the norite was washed with 1 ml distilled H_2O per 0.1 μmole, spun again after decantation and eluted with 1 ml 50 % ethanol, 0.05 M in NH_3. After 5 min. the mixture was spun and elution was repeated with 1 ml ethanol. After evaporation of the combined eluates to a small volume, the sample was ready for chromatography. The washing of the norite with H_2O is particularly important as it removes inorganic salts, which otherwise considerably delay the drying of the spots when they are deposited on the paper.

Chromatography was carried out in ethanol-ammonium acetate solvent at pH 7.5 [24]. In this solvent UMP, UDP, UDPG and UDPAG are easily separated from one another, while G-1-P and UDPG run together. In experiments where it was necessary to separate those two compounds, the samples were chromatographed in ethanol ammonium acetate at pH 4 [24]. The nucleotide spots were localized on the paper by the "mineralight" lamp, and when desired, the chromatogram was scanned in the Beckman at 260 mμ according to the method of Leloir and Paladini [23] on strips of 1 cm width, cut out of the paper chromatogram. Impregnation of the paper with liquid paraffin was found unnecessary. In the cases, where radioactive material had been applied, the paper strips were scanned with respect to radioactivity in a Geiger-Müller counter; or the spots were cut out and eluted with H_2O, followed by estimation of ultraviolet absorption and counts/ml in the eluate.

RESULTS

Purification of enzyme

Purification procedure included the following steps: Extraction, ammonium sulphate fractionation, precipitation with protamine, ethanol fractionation and finally another fractionation with ammonium sulphate. Cuvette assays as described in Methods were the basis for activity measurements during the purification procedure. It should be mentioned that in the crude extract the presence of nucleotide pyrophosphatase [25] made activity measurements

very inaccurate. In such extracts estimations of the activity were made by comparing the amount of G-1-P formed with and without addition of pyrophosphate.

Extraction. 20 g of dried brewer's yeast (Kongens Bryghus) were autolyzed and extracted by shaking with 40 ml 0.07 M $(NH_4)_2HPO_4$ for 18 hours at 20° C. The extract was spun, whereby 23 ml of filtrate was obtained. To the supernatant was added 15.6 ml saturated ammonium sulphate to make the solution 40 % saturated. After 30 min. at 0° C a small precipitate was separated by centrifugation, and to the supernatant was added 19 ml saturated ammonium sulphate to make the solution 60 % saturated. After half an hour at 0° C a large precipitate was collected by centrifugation. Further addition of ammonium sulphate to the supernatant yielded only a slight precipitate.

The precipitate obtained at 40—60 % ammonium sulphate saturation was dissolved at zero in 25 ml 0.015 M acetate buffer, pH 6.3, and dialyzed for half an hour against tap water. To the clear solution (27 ml) was added 2 ml of a 1 % solution of protamine sulphate (salmine). After 1 hour at 2° C a small precipitate was removed by centrifugation while the main part of the uridyl transferase activity remained in the supernatant. The clear supernatant was cooled to —2° C and fractionated with ethanol. The temperature was gradually lowered to —8° C during dropwise addition of 50 % ethanol. The fractions precipitated between 20—24 %, between 24—28 % and finally between 28—31 % ethanol were collected by centrifugation at —10° C in a previously cooled centrifuge.

The ethanol precipitated fractions were kept at —20° C overnight. By this treatment, the ethanol, adhering to the precipitate and to the walls of the centrifuge tubes, evaporates completely. Traces of ethanol may otherwise cause considerable denaturing of the protein to be dissolved. Next morning the fractions were dissolved in cold distilled water. The fraction precipitated between 20—24 % ethanol usually contained a small amount of insoluble material, which was discarded by centrifugation. The other fractions yielded clear solutions.

The highest activity was usually found in the fraction precipitated at 20—24 % ethanol. To the clear solution (4 ml) was added 6 ml saturated ammonium sulphate to make the solution 60 % saturated. The precipitate was collected by centrifugation and extracted three successive times with 2.5 ml aliquots of decreasing ammonium sulphate concentrations, which were adjus-

Table 1. Purification of UDPG pyrophosphorylase.

		Vol. of fraction	Total activity	Yield %	Specific activity
Crude extract		23 ml	4 055		0.3
Amm. sulphate fraction	40—60 %	28 »	3 360	82.8	6.3
Ethanol fraction I	20—24 %	6.4 »	1 640	40.4	11.0
» » II	24—28 %	3 »	241	5.9	5.5
» » III	28—31 %	2 »	121	2.9	5.8
2nd amm. sulphate fraction					
a	40—46 %	0.5 »	196	4.8	18.3
b	46—50 %	2.5 »	693	15.3	40.7
c	50—56 %	2.5 »	618	15.0	77.2

ted to pH 7.5 with ammonia. The fraction obtained at 50—56 % saturation was the most active. It was more than 250 times as active as the crude yeast extract and represented an overall yield of 15 % (Table 1). (The concentrations of ammonium sulphate in these fractions were estimated by conductivity measurements).

The product thus obtained was free of G-6-P dehydrogenase, of 6-phosphogluconic acid dehydrogenase, of hexokinase and of phosphoglucomutase. It was stored as ammonium sulphate precipitate at —20° C.

A number of preparations were carried out from the same batch of dry yeast with approximately the same yield and enzyme activity. Other samples of dried yeast obtained from the same brewery, however, resulted in preparations with lower activities and yield.

Properties of enzyme

Stability. The purified preparation is extremely unstable in dilute solutions. Attempts to stabilize the solution by addition of crystalline bovine serum albumin (3 mg/ml) failed. It was later found that the best way of storing the enzyme was to keep the final ammonium sulphate precipitate at —20° C. Kept in this way a 3 months' old preparation still contained 60—75 % activity.

Equilibrium. The UDPG pyrophosphorylase reaction is reversible, as has earlier been shown by means of ^{32}P-labelled UTP or ^{32}P-labelled G-1-P [26, 6].

Balance studies of the reaction were done by incubating equimolar amounts of UDPG and PP with the purified enzyme and assaying the G-1-P formed in aliquots taken out at different time intervals. For this purpose a G-6-P dehydrogenase preparation, free of UDPG pyrophosphorylase, as described in Methods, was used.

The experimental incubation mixture contained 2 μmoles UDPG, 2 μmoles PP, 0.08 mg/ml of enzyme, and 0.05 M Tris, pH 7.4. Total volume was 8 ml. Aliquots of 400 μl were taken out at different times, and the reaction was stopped by heating the tube 10 sec. in boiling water, followed by rapid cooling. The protein precipitate was allowed to settle, before 300 μl of the digest were diluted with 600 μl Tris, 0.05 M, pH 7.3, in a 1 ml Beckman cuvette and analyzed for G-1-P by addition of phosphoglucomutase, cysteine, TPN and G-6-P dehydrogenase. When readings at 340 mμ were constant, indicating that all the G-1-P was used, UDPG pyrophosphorylase and more PP were added to estimate the remaining UDPG content of the digest.

In Fig. 2 is shown the equilibrium curve for the reaction. From this it may be concluded that the reaction stops at approximately 45 % conversion, yielding an equilibrium constant close to 1. Correspondingly the UDPG content of the enzyme digest decreases and reaches a final constant value of about 52 % of the initial UDPG concentration. This indicates, as could be expected, that the pyrophosphorolytic reaction involves no appreciable thermodynamic changes.

pH dependence. In the determination of the pH optimum of the UDPG pyrophosphorylase, the spectrophotometric method could not be used as this assay involves other enzyme systems, whose pH optima differ from that of the

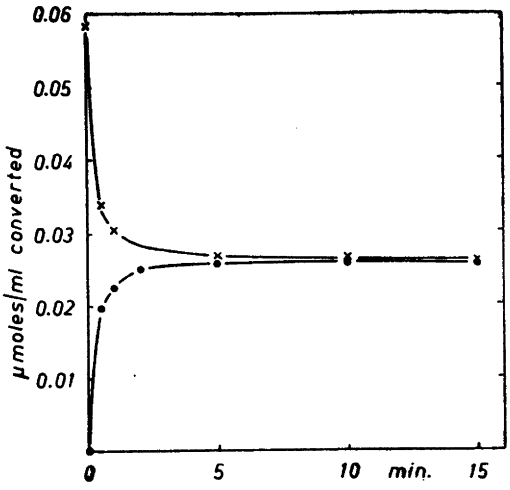

Fig. 2. Attainment of equilibrium in the reaction UDPG + PP ⇌ UTP + G-1-P.
Experimental data as described in text. ●–●–● formation of G-1-P. × — × — × disappearance of UDPG.

pyrophosphorylase. Experiments were therefore carried out in two stages by a method similar to that used in determination of the equilibrium constant.

A double series of samples with buffers of varying pH, each containing 0.1 µmole of UDPG and 0.1 µmole of pyrophosphate were incubated with the enzyme, in the first series for 30 sec. and in the second for 2 and a half min. The reaction was stopped by heating, and analysis of the G-1-P formed in the 2 min. interval was carried out according to the method described in equilibrium experiments.

In Fig. 3 the reaction velocities in the different buffers are plotted against the pH of the solution. It is seen that the reaction has a broad, but distinct optimum between pH 6.5 and pH 8.

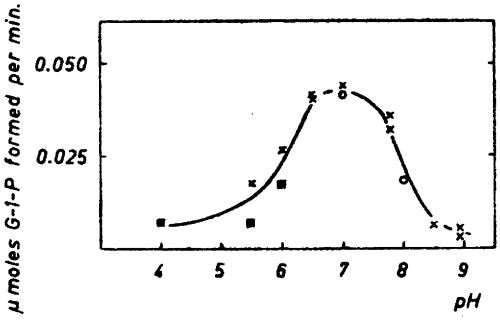

Fig. 3. Influence of pH on the UDPG pyrophosphorylase reaction.
The buffers used were 0.05 M. O—O—O phosphate buffer. × — × — × Tris buffer.

Mg activation. The Mg^{++} requirement was investigated by a method similar to the pH dependence measurements of the UDPG pyrophosphorylase and is shown in Fig. 4. The reaction rate was optimal at a concentration of 2×10^{-3} M Mg^{++}.

Michaelis constant. The Michaelis constant for the UDPG was found to be 0.7×10^{-4}. The constants for the other substrates have not been measured.

Lag period. The spectrophotometric method which is ordinarily used in the assay of UDPG shows a characteristic lag period (Fig. 1), which can be overcome only by addition of very large amounts of the UDPG pyrophosphorylase. Addition of excess of phosphoglucomutase and G-6-P dehydrogenase does not influence this phenomenon. For closer investigation of the lag period an experiment was set up to compare the velocity curve, obtained by the spectrophotometric assay, with a velocity curve derived from G-1-P determination on aliquots taken out with short intervals in the initial stage of the reaction. For this experiment was used a UDPG solution freed of G-1-P by paper chromatography at pH 3.9 and subsequent elution of the UDPG from the paper.

The amounts of enzyme and substrate were so adjusted that the rate of velocity was approximately the same in the two experiments and Fig. 5 shows the experimental results; it is seen that in the case where the rate of velocity is determined on aliquots of the reaction mixture there is no sign of lag period. The occurrence of a lag period in the spectrophotometric assay is most likely explained by the relatively large K_m for the G-1-P in the phosphoglucomutase reaction (see Discussion).

Influence of other factors. The rate of the pyrophosphorolytic reaction as followed by the spectrophotometric assay was found to be unaffected by the addition of sodium fluoride to a concentration of 0.05 M. Also versene (0.01 M) had no inhibitory effect. Uridine diphosphoglycosyl compounds are shown to accumulate in penicillin-treated *Staphylococcus aureus* cells [27, 28], and for this reason penicillin was tested as a possible inhibitor for the pyrophosphorylase reaction, but it was found that even in concentrations up to 10 mg/ml this substance did not materially influence the reaction.

UMP and UDP were also tested for a possible inhibitory effect, but with negative results. Cysteine does not inhibit the reaction.

Specificity of the reaction. Crude yeast and liver extracts have been shown to contain a number of pyrophosphorylases, acting on different substrates. The purified UDPG pyrophosphorylase has been tested for activity towards several nucleoside diphosphate compounds, but it seems to be quite specific with respect to the carbohydrate group as well as the nucleoside moiety. UDPAG is not attacked and neither is GDPM; also DPN is left untouched.

The reaction mechanism of UDPG pyrophosphorylase

The UDPG pyrophosphorylase belongs to a group of enzymes, which may be classified as uridyl transferases. In this particular case G-1-P and PP function as donor and acceptor, respectively, of the uridyl group.

If, in group transfer reactions, the enzyme is considered as an acceptor of

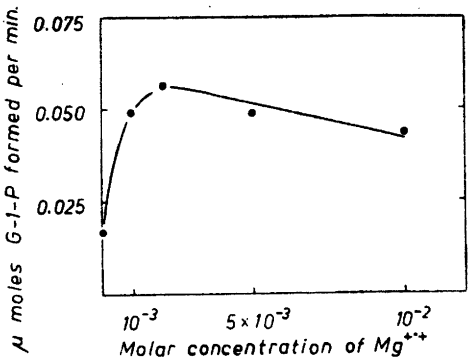

Fig. 4. Influence of Mg^{++} on the UDPG pyrophosphorylase reaction.

anhydride groups [29, 9, 30] the UDPG pyrophosphorylase reaction may be visualized as a two step reaction according to the following scheme:

$$\begin{array}{lll} \text{UDPG} + \text{Enz} \rightleftharpoons \text{UMP}\sim\text{Enz} + \text{G-1-P} & a & \\ \text{UMP}\sim\text{Enz} + \text{PP} \rightleftharpoons \text{UTP} + \text{Enz} & b & \end{array} \qquad \text{I}$$

This formulation postulates the formation of a uridyl-enzyme compound.

Another possibility is that the reaction follows a three step pattern (*cf.* Kalckar [9]) with the following sequence of reactions:

$$\begin{array}{l} \text{UDPG} + \text{Enz} \rightleftharpoons \text{UMP} + \text{G-1-P}\sim\text{Enz} \\ \text{G-1-P}\sim\text{Enz} + \text{PP} \rightleftharpoons \text{G-1-P} + \text{PP}\sim\text{Enz} \\ \text{PP}\sim\text{Enz} + \text{UMP} \rightleftharpoons \text{UTP} + \text{Enz} \end{array} \qquad \text{II}$$

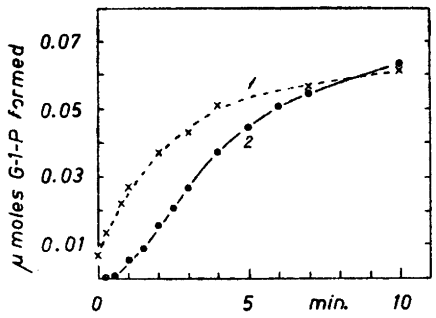

Fig. 5. Comparison between spectrophotometric and direct registration of the UDPG pyrophosphorylase reaction.

Experiment 1: 0.9 μmoles UDPG, 10 μmoles PP, 5 μl purified UDPG pyrophosphorylase (0.9 mg protein/ml), 0.05 M Tris, pH 7.3, to a volume of 1.3 ml. Aliquots of 100 μl taken out at different times and assayed for G-1-P content.

Experiment 2: 0.5 μmoles UDPG, 25 μl mutase (3 mg protein/ml), 25 μl cystein (10 mg/ml), 10 μl G-6-P dehydrogenase, 20 μl TPN (0.01 M), and 0.05 M Tris, pH 7.3, to a volume of 700 μl. The reaction was started by addition of 2.5 μl purified UDPG pyrophosphorylase and 10 μl PP, 0.1 M. Readings were at same time intervals as in experiment 1.

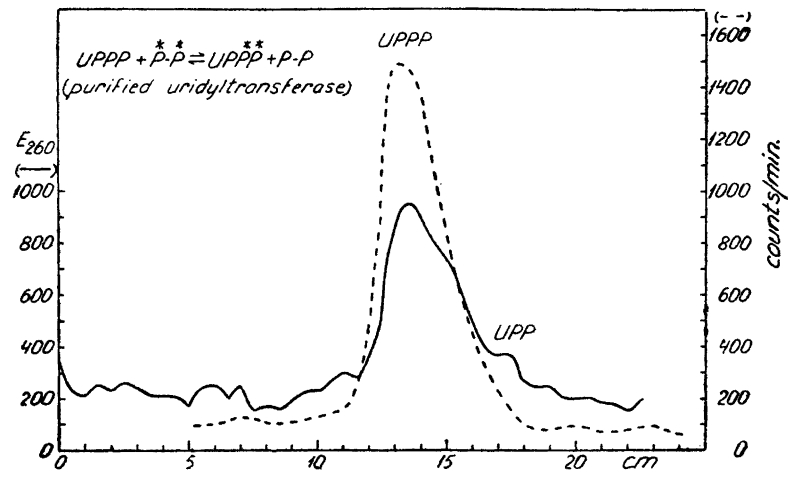

Fig. 6. Exchange experiment with UTP and $^{32}P^{32}P$.

0.25 μmoles UTP, 0.5 μmoles $^{32}P^{32}P$ (96 000 cts/min/μmole), 100 μl purified UDPG pyrophosphorylase (1.6 mg protein/ml), and Tris to a volume of 1.1 ml were incubated 1 hour. After deproteinization the nucleotides were adsorbed on norite and chromatographed. The chromatogram was scanned in the Beckman and counted in a Geiger-Müller counter.

Attempts to decide between the two possibilities were made by carrying out some isotope exchange experiments, although only indirect evidence for either of the two mechanisms can be obtained by such methods.

The following three labelled compounds were available: ^{14}C-labelled UMP [15], ^{32}P-labelled G-1-P and ^{32}P-labelled pyrophosphate. It is clear from the reaction schemes written above that if scheme I is valid, equation *a* would effect an incorporation of G-1-^{32}P in UDPG, if these two substances were incubated with the enzyme in the absence of PP; correspondingly equation *b* would cause an incorporation of $^{32}P^{32}P$ in UTP, if the two substances were incubated with the enzyme.

Table 2. Exchange experiments with ^{14}C-labelled UMP.

0.25 μmole of UTP or UDPG were incubated 60 min. with 0.1 μmole of ^{14}C-UMP (32 100 cts/min/μmole) in the presence of purified UDPG pyrophosphorylase. The reaction mixture was acidified, adsorbed on norite and chromatographed in ethanol-ammonium acetate at pH 7.5. The ultraviolet absorbing spots corresponding to UDPG, UTP and UMP were eluted and counted.

A control, containing 0.25 μmole UTP and 0.5 μmole. $^{32}P^{32}P$ (30 000 cts/min/μmole) together with the enzyme, was run simultaneously.

	cts/min/μmole		
	UTP	UDPG	UMP
UTP + ^{14}C—UMP + Enzyme	22	—	31 200
UDPG + ^{14}C—UMP + Enzyme	—	10	27 700
UTP + $^{32}P^{32}P$ + Enzyme	4 540	—	—

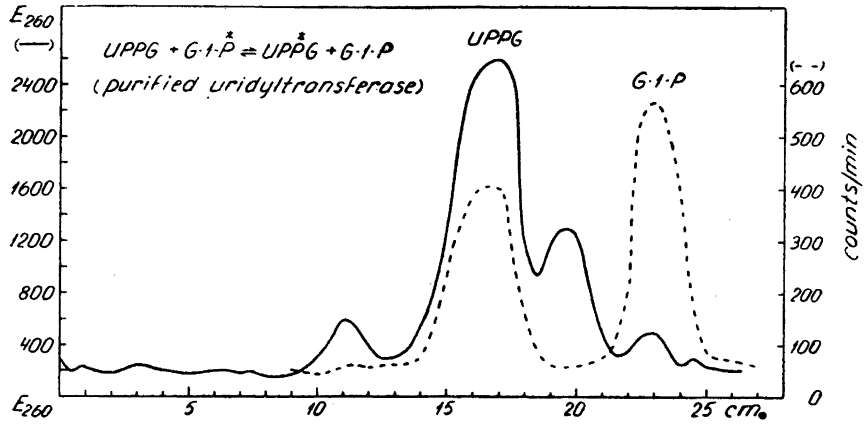

Fig. 7. Exchange experiment with UDPG and G-1-^{32}P.

0.7 μmoles UDPG, 1 μmole G-1-^{32}P (20 000 cts/min/μmole), 25 μl purified UDPG pyrophosphorylase (1.6 mg protein/ml) and 0.05 M Tris, pH 7.3, to a volume of 1.2 ml were incubated 1 hour. The reaction mixtures were treated as described in Fig. 6.

If, on the other hand, ^{14}C-labelled UMP could be incorporated in UDPG as well as in UTP in the absence of PP and G-1-P, respectively, this would indicate that the UDPG pyrophosphorylase might follow scheme II; in this case the enzyme could not be classified as a true uridyl transferase.

Enzyme preparations from brewer's yeast and of different purity were incubated with 0.2—0.5 μmoles of substrate together with the corresponding labelled uridyl acceptor. Incubation periods of 1—2 hours were chosen as it was found that further incubation destroyed the enzyme. After deproteinization of the reaction mixture the nucleotides were adsorbed on norite and chromatographed on paper. The ultraviolet absorbing spots were eluted and counted, or the chromatogram was scanned in the Beckman at 260 mμ and in a Geiger-Müller counter in the usual way. In some cases also autoradiography was applied.

In such experiments it was found that when a purified preparation of the UDPG pyrophosphorylase was incubated with UDPG and G-1-^{32}P or with UTP and ^{32}P^{32}P, a rapid exchange of G-1-^{32}P into UDPG and of ^{32}P^{32}P into UTP took place. If the same enzyme preparation was incubated with

Table 3. Incorporation of ^{32}P^{32}P into different nucleoside triphosphates.

Samples containing 0.2 μmole of GTP, ATP and UTP were incubated 60 min. with 0.5 μmole ^{32}P^{32}P (450 000 cts/min/μmole) and 1) a crude extract of *Sacch. fragilis*, 2) purified UDPG pyrophosphorylase. Reaction mixture adsorbed on norite, chromatographed and counted as described in the text.

	cts/min/μmole		
Enzyme applied	GTP	ATP	UTP
Extract of *S. fragilis*	28 100	26 700	30 000
Purified UDPG pyrophosphorylase	0	1 240	57 000

^{14}C-labelled UMP and either UDPG or UTP, no incorporation of this compound in either of the nucleotides could be detected. The results are presented in Figs. 6 and 7 and in Table 2. Control experiments where the reactants were incubated together in absence of enzyme showed that no non-enzymatic exchange occurs between these compounds.

That the incorporation is effected by the UDPG pyrophosphorylase and not by contaminating enzymes is supported by the fact that the incorporation rate of $^{32}P^{32}P$ into UTP rises with increasing purity of the enzyme (Fig. 8). Moreover, the incorporation of labelled PP into UTP is almost specific for the purified UDPG pyrophosphorylase, whereas less purified enzyme preparations also catalyze a corresponding incorporation into GTP and ATP (Table 3).

The observations made in these experiments all seem to favour scheme I in the argument above, and so far no evidence has appeared to support scheme II. All attempts, however, to isolate a uridyl enzyme compound from a digest consisting of the purified enzyme with either ^{14}C-labelled UDPG or ^{14}C-labelled UTP, have been fruitless.

DISCUSSION

The UDPG pyrophosphorylase belongs to a class of enzymes which has been named nucleotidyl transferases [9], and of which several are already known [4, 6, 7]. With respect to equilibrium, Michaelis constant, and the influence of factors like magnesium and fluoride ions the UDPG pyrophosphorylase shows similarities with the DPN pyrophosphorylase, described by Kornberg [4].

The characteristic lag period, which is always observed, when the reaction is followed spectrophotometrically, has been investigated more closely, and the results obtained indicate that the phenomenon does not originate from the UDPG pyrophosphorylase reaction itself, but must be ascribed to the complexity of the assay system. The phosphoglucomutase reaction is inhibited, presumably competitively, by salts [31], and as the assay mixture contains relatively large amounts of anions, it is reasonable to believe that the value of the Michaelis constant for G-1-P is considerably increased in this medium. Hence the conversion of G-1-P, formed by the UDPG pyrophosphorylase reaction, to G-6-P proceeds very slowly until the G-1-P concentration rises above the K_m of the phosphoglucomutase reaction, and the rate of velocity gradually attains the proper value.

In recent years investigators have tended to consider the enzymatic substitution reactions as analogous to the non-enzymatic substitution reactions, known from studies of organic chemical mechanisms. Such considerations have been advanced by Koshland [29], who shows that a few basic mechanisms in a number of cases can explain a variety of experimental data, obtained chiefly in exchange experiments with enzymes and labelled substrates.

In an attempt to apply considerations of this kind to the uridyl transferase reaction a number of isotopic exchange experiments have been carried out with the UDPG pyrophosphorylase. The results obtained agree readily with the assumption of an intermediate uridyl-enzyme complex in which case the transfer of the uridyl group is believed to proceed through a "double displacement mechanism" (*cf.* Koshland) as depicted in scheme I, p. 1531. It should

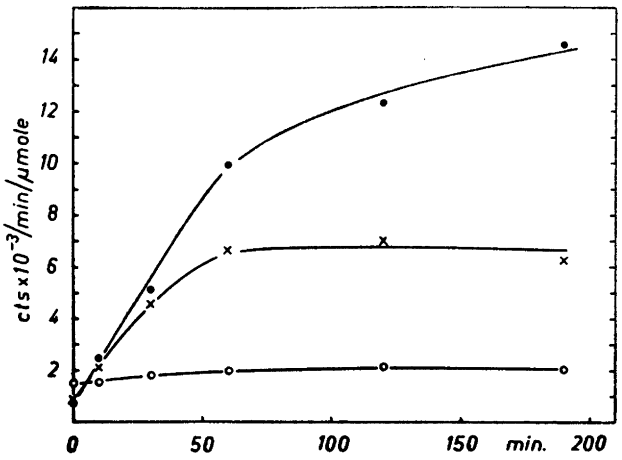

Fig. 8. Incorporation of $^{32}P^{32}P$ into UTP, catalyzed by enzyme preparations of different purity.

Three set of samples containing 1 ml Tris, 1 μmole $^{32}P^{32}P$ (90 000 cts/min/μmole), and 0.5 μmole UTP were incubated with UDPG pyrophosphorylase of different purity. The reaction was stopped at the times indicated on the curve, and the reaction mixtures were treated with norite, chromatographed and counted as described in the text.

●–●–●	0.2	mg	enzyme,	specific activity	5
×–×–×	2.2	»	»	»	0.5
O–O–O	4.2	»	»	»	0.1

be added, though, that this interpretation of the exchange data must be taken with reservation as long as a more direct proof of the hypothesis, *e. g.* the isolation of the uridyl-enzyme complex, has not been obtained.

Trucco [32] has reported the incorporation of ^{14}C-labelled G-1-P in UDPG when these two substances are incubated with dialyzed extracts of *Saccharomyces fragilis*. This incorporation is ascribed to the combined effect of the following two enzymatic reactions, both catalyzed by extracts of *S. fragilis* [33, 34].

(1) Gal-1-P + UDPG ⇌ UDPGal + G-1-P (non pyrophosphorolytic uridyl transferase)

(2) UDPGal ⇌ UDPG (galacto-waldenase)

The fact that the purified UDPG pyrophosphorylase catalyzes a similar exchange (G-1-^{32}P into UDPG) has in the present investigation been explained by the formation of an uridyl-enzyme compound in the uridyl transferase reaction. This assumption makes the action of galacto-waldenase unnecessary to effect the incorporation of labelled G-1-P into UDPG, and such an incorporation, catalyzed by extracts of *S. fragilis*, may with equal reason be explained by the action of either the UDPG pyrophosphorylase or the non-pyrophosphorolytic uridyl transferase, both present in dialyzed extracts of *S. fragilis*.

Acknowledgements. I wish to thank Dr. H. M. Kalckar for inspiring suggestions and for many stimulating discussions.

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Note on the Transphosphorylation Reaction between Uridine Monophosphate and Adenosine Triphosphate

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Spectrophotometric demonstration of uridine diphosphate is described. By use of adenosine triphosphate, ^{32}P-labelled in the terminal phosphate group, it is shown that the phosphorylation of uridine monophosphate by adenosine triphosphate, as catalyzed by yeast extract, is a transfer of one phosphate group, yielding uridine diphosphate and adenosine diphosphate.

A transphosphorylating reaction between mononucleotides and trinucleotides has at the same time been observed in several laboratories [1-4], and the appropriate enzymes have been demonstrated in yeast as well as in liver. So far it has been established that uridine-, cytidine-, guanosine-, and adenosine phosphates participate in reactions of this kind, but the specificity of the enzyme or enzymes is not yet known. In the following is described the demonstration of the following reaction *:

$$UMP + ATP \rightarrow UDP + ADP$$

It was noticed that incubation of UMP and ATP with yeast extract resulted in formation of UDP and UTP, which could be separated by ionophoresis from other nucleotides present. The ionophoresis was carried out in 0.02 M citrate buffer, pH 4.5, and a voltage of 220 volts was applied for 22 hours at 4° C. By this procedure the uridine di- and triphosphates are easily separated from the corresponding adenine nucleotides (*cf.* Ref. [5]).

UDP was identified by reversing the transglycosidic reaction, described by Leloir and Cardini [6], by which sucrose is synthesized from UDPG and fructose:

$$UDPG + fructose \rightleftharpoons UDP + sucrose$$

* The following abbreviations are used: UMP for uridine monophosphate, ATP for adenosine triphosphate, UDP for uridine diphosphate, ADP for adenosine diphosphate, UTP for uridine triphosphate, UDPG for uridine diphosphoglucose, TPN for triphosphopyridine nucleotide, G-6-P for glucose-6-phosphate, G-1-P for glucose-1-phosphate and PP for inorganic pyrophosphate.

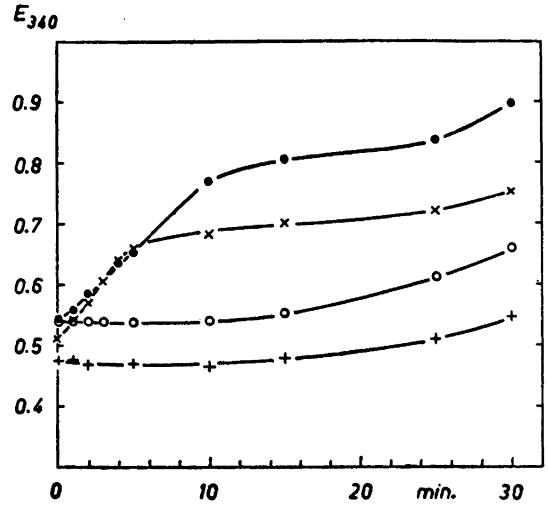

Fig. 1. Spectrophotometric demonstration of UDP.

The complete reaction mixture (in a 1 ml quartz cuvette) contained 1 ml 0.05 M tris-hydroxymethyl amino methane, HCl, pH 7.3, 200 µl wheat germ enzyme (0.3 mg protein/ml), 100 µl sucrose (0.2 M), and 0.2 µmole UDP. The mixture was incubated 20 min. at 30° C, and after cooling to room temperature the following were added (*cf.* Ref.[7]): 25 µl phosphoglucomutase (3 mg protein/ml), 25 µl cysteine (10 mg/ml), 10 µl G-6-P dehydrogenase, containing also the UDPG pyrophosphorylase, 20 µl TPN (0.01 M), and finally 10 µl PP (0.1 M) to start the reaction, which was followed spectrophotometrically at 340 mµ.

+—+—+ UDP omitted, O—O—O sucrose omitted,
×—×—× known UDP added, ●—●—● UDP from paper eluate (see text).

The enzyme was prepared from wheat germ and purified to the extent described by Leloir. The resulting enzyme solution contained 0.3 mg protein per ml and was stable for several weeks when kept at —20° C. By incubating UDP with this enzyme and excess sucrose at pH 7.0, UDPG is formed, and by addition of TPN, UDPG pyrophosphorylase, G-6-P dehydrogenase, phosphoglucomutase and pyrophosphate the UDPG could be demonstrated spectrophotometrically at 340 mµ [7].

In Fig. 1 are shown the curves obtained with authentic UDP and with paper eluate of the suspected UDP. Controls omitting either of the reactants were run simultaneously. It should be emphasized that this assay cannot be used for quantitative determination of UDP, as the equilibrium of the reaction lies too far towards sucrose.

The UTP formed during the incubation of ATP and UMP with the yeast extract was identified together with catalytic amounts of ADP, according to the spectrophotometric method of Berg and Joklik [5].

It was left to decide whether the reaction was a transfer of pyrophosphate with subsequent partial dephosphorylation of UTP to UDP, or a transfer of one phosphate group to form UDP which then by the action of nucleoside diphosphokinase was phosphorylated to UTP. For this purpose ATP, labelled

Table 1. Transfer of labelled phosphate from ATP to UMP.

The reaction mixture contained, in a total volume of 1 ml, 0.5 μmole ATP, 8.1×10^3 cts/min/μmole, 0.6 μmole 5-UMP, 50 μl enzyme solution (8 mg protein/ml), 0.005 μmole $MgCl_2$, 0.05 M tris-hydroxymethyl amino methane, HCl, pH 7.3.

Nucleotide analyzed	cts/min/μmole
ATP	7 250
UDP	7 780
ADP	0

in the terminal phosphate group, was prepared from UDPG, ^{32}P-labelled pyrophosphate and ADP, according to the equations below: (UPPP stands for UTP, APPP for ATP, UPP for UDP and APP for ADP)

$$UDPG + {}^{32}P{}^{32}P \rightarrow UP{}^{32}P{}^{32}P + G\text{-}1\text{-}P$$ (UDPG pyrophosphorylase [7])

$$UP{}^{32}P{}^{32}P + APP \rightarrow UP{}^{32}P + APP{}^{32}P$$ (nucleoside diphosphate kinase [5])

As source of enzymes a G-6-P dehydrogenase preparation (LePage and Mueller [8]) was used, which contained abundant amounts of the UDPG pyrophosphorylase as well as of nucleoside diphosphate kinase. The ATP was isolated from the reaction mixture by adsorption on norite and elution with 50 % ethanol, followed by paper chromatography and elution of the ATP spot [7].

The labelled ATP was incubated with UMP in presence of the yeast enzyme. After the reaction was stopped, the nucleotides were isolated by paper ionophoresis, eluted and analyzed with respect to concentration and radioactivity. The results are presented in Table 1 and show clearly, that the reaction involves a transfer of only the terminal phosphate group of ATP to UMP, resulting in the formation of UDP with the same activity as the initial ATP.

Attempts to demonstrate, spectrophotometrically, by means of Leloir's enzyme and UDPG pyrophosphorylase, the following sequence of reactions:

$$UTP + UMP \rightleftharpoons 2\ UDP \qquad (1)$$
$$UDP + \text{sucrose} \rightarrow UDPG + \text{fructose}$$
$$UDPG + PP \rightarrow UTP + G\text{-}1\text{-}P$$

were negative, although reaction (1) has been reported to proceed in analogy with the myokinase reaction, in the presence of yeast extract [1].

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Short Communications

A Note on the Connection between Viscosity and Structure in the System CaO-SiO_2

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In a recent study of viscosity in the system $CaO-SiO_2$ Bockris and Lowe [1] have tried to interpret the anion structure in the region metasilicate-orthosilicate in terms of the variation of the energy of activation with the composition. According to them orthosilicate anions consist of discrete SiO_4^{4-} entities, which are known from X-ray data of solid silicates and at the stoichiometric composition $3CaO{\cdot}2SiO_2$ the anions have the composition of $Si_2O_7^{6-}$. As seen from the phase diagram [2] this is not very likely, because this compound melts incongruently. On further addition of SiO_2 polymerization should continue, the flow unit being silicate chain ions. But at 50 mole-% CaO an anion $Si_3O_9^{6-}$ should probably be present and represents the meta-anion. This latter form is in agreement with what is found in the mineral wollastonite ($CaSiO_3$) and the results from the determinations of freezing point depression in the system calcium metasilicate-calcium fluoride [3].

It seems probable from the phase diagram of the system $CaO-SiO_2$ that not very many types of compounds can exist in the molten state and under no circumstance as many as Bockris and Lowe have concluded. The simplest is to assume that in the part of the system under discussion we only have discrete anions of metasilicate of the composition $Ca_3Si_3O_9$ and orthosilicate Ca_2SiO_4. In Fig. 1 the mole fractions of $Ca_3Si_3O_9$ have been plotted against

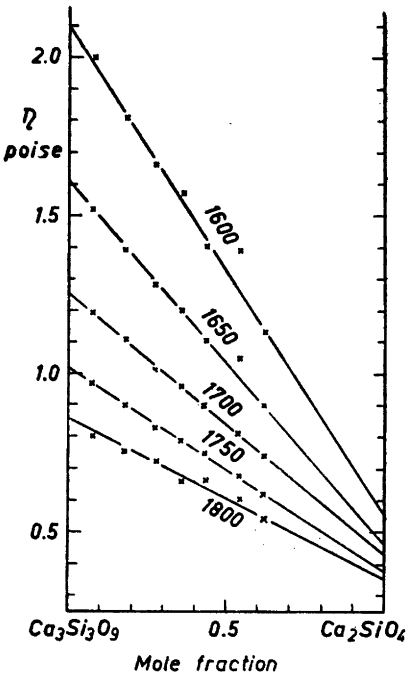

Fig. 1

the viscosities determined by Bockris and Lowe. It is seen that the viscosity is a linear function of $x_{Ca_3Si_3O_9}$. In the ideal case when we have a mixture of two liquids and there is no interaction between them the viscosity is a linear function of the relative composition [4]. It also seems very likely that there will not be any appreciable heat of mixing between two silicates of the same cation. In Table 1 two different sets of equations for the isotherms are recorded, one where the viscosity is a function of the composition and the other

Table 1.

°C	η	η_{rel}
1 550	$2.18 \cdot x + 0.720$	$3.02 \cdot x + 1$
1 600	$1.55 \cdot x + 0.555$	$2.79 \cdot x + 1$
1 650	$1.20 \cdot x + 0.480$	$2.33 \cdot x + 1$
1 700	$0.83 \cdot x + 0.429$	$1.94 \cdot x + 1$
1 750	$0.62 \cdot x + 0.391$	$1.59 \cdot x + 1$
1 800	$0.46 \cdot x + 0.375$	$1.23 \cdot x + 1$

x= mole fraction of $Ca_3Si_3O_9$.

where the relative viscosity is a function of the composition. In the latter set it is seen that the coefficients of $x_{Ca_3Si_3O_9}$ are smaller for higher temperatures and are a linear function of the temperature (Fig. 2).

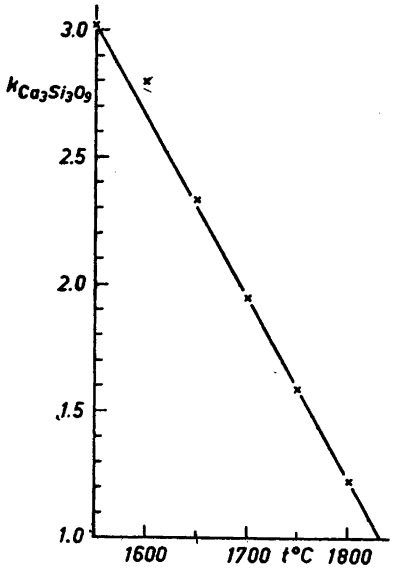

Fig. 2

From the above arguments it can be concluded that very simple relations must exist in the regions of the system under consideration and that it is very probable that essentially only a metasilicate of the composition $Ca_3Si_3O_9$ and an orthosilicate Ca_2SiO_4 exist in the molten state.

1. Bockris, J. O'M. and Lowe, D. C. *Proc. Royal Soc. London* **226 A** (1954) 423.
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Isolation of Sialic Acid from Gangliosides

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As far back as 1938 one of us [1] called attention to the close agreement in certain qualitative reactions between gangliosides and a substance some years earlier isolated from bovine submaxillary mucin [2] and later named sialic acid [3]. In 1941 Klenk [3] obtained from the methanolysate of gangliosides the monomethoxy derivative of a split product called neuraminic acid which showed the characteristic colour reactions of sialic acid. In 1952 Blix *et al.*[3] pointed out that the neuraminic acid might have been formed during the isolation procedure by deacetylation of sialic acid, which at that time had been with certainty demonstrated as a component of bovine submaxillary mucin only. The unchanged sialic acid from this material contains one N-acetyl and one very labile O-acetyl [2,3], whereas the neuraminic acid has no acetyl group. The difference in elementary composition between the two substances was reasonably in agreement with a deacetylation. This view was further supported by the isolation of methoxy-neuraminic acid from a methanolysate of submaxillary mucin (Klenk and Lauenstein [6]). Employing a milder procedure Klenk and Faillard [7] later obtained from bovine submaxillary mucin a split product with an elementary composition and an acetyl content corresponding to that calculated for monoacetyl neuraminic acid. This product could by treatment with methanol-HCl be transfor-

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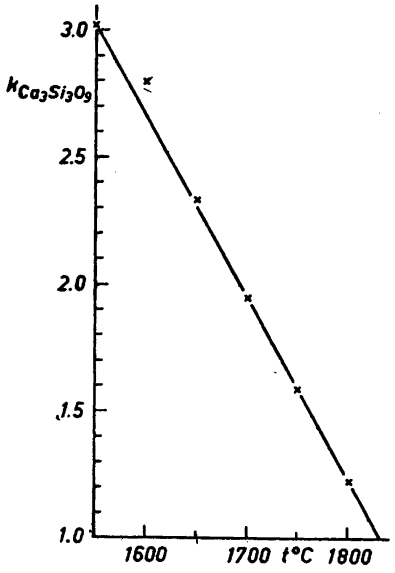

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med into methoxyneuraminic acid *. By the same means we have transformed bovine as well as porcine sialic acid into methoxy-neuraminic acid [8]. The close relationship between sialic acids and neuraminic acid is thus well substantiated. Recently Svennerholm [9] announced that he had been able to isolate from gangliosides a crystalline substance which gave the direct Ehrlich reaction of sialic and neuraminic acids, but, in contra-distinction to the latter, a negative ninhydrin reaction.

We have now succeeded in isolating sialic acid from gangliosides by a very simple method. The yield was 20 mg of recrystallized acid from 0.65 g of ganglioside. The X-ray powder photograph of the acid was identical with that of the sialic acid which is obtained from sheep submaxillary mucin as well as from a number of human glycoproteins and from ovomucin [10].

Experimental. The gangliosides were prepared from normal human brains in the main according to Klenk [11]. The substance was however dialysed against water and freed from cations by thorough treatment with a cation exchange resin. It contained 2.35 % N, 10.4 % hexosamine, and 27 % sialic acid (colorimetrically determined). It was free from phosphorus. 0.65 g of the ganglioside was dissolved in 10 ml of water and dialysed firstly against 0.005 N hydrochloric acid at 4° C for 24 hours, and then against distilled water until free from chlorine. The solution, which had a strongly acid reaction, was placed in a cellophane bag and dialysed against 100 ml of water at 37° C for 24 hours. A few drops of toluene were added to the fluids. The dialysate was freeze-dried. The dialysis was repeated in the same way on five subsequent days, or as long as appreciable amounts of Bial-positive material appeared in the dialysate. Obviously the acid reaction produced by the ganglioside itself (initially about pH 2.5) was sufficient to split off most of the sialic acid, as the product remaining in the inner fluid at the end of the experiment contained only 7 % of sialic acid. The ease with which the sialic acid was split off indicates that it is attached at least partly as an end group in the ganglioside.

The lyophilized residues were taken up in methanol, and the extract evaporated *in vacuo*. The partly crystalline residue was washed with ether, and dissolved in a minimum amount of water. After addition of a few ml of methanol, ether was added in small portions as long as amorphous precipitates were formed. These contained sialic acid together with other material. They were removed by filtration, and not further examined. Petroleum ether was added to the filtrate until a faint opalescence appeared. On standing at room temperature the sialic acid crystallized in needles. The crystalline material was washed with cold methanol, dissolved in a little water and methanol, and recrystallized by addition of ether and petroleum ether. (Found: N 4.40; 4.48. Calc. for $C_{11}H_{19}NO_9$: N 4.53). Quantitative determinations with Bial's and Ehrlich's reagents [12] and paper-chromatographic analysis

* Apparently the same substance has been obtained by us from bovine submaxillary mucin which had been dialysed against weak HCl. The substance showed the same X-ray diffraction pattern as the sialic acid from sheep submaxillary mucin [5]. This form of sialic acid contains only an N-acetyl. Obviously the labile O-acetyl was also split off during the isolation procedure employed by Klenk and Faillard. As it cannot be excluded that treatment with methanol-HCl at 105° C brings about intramolecular changes of the very unstable sialic acids, the name 'acetyl neuraminic acid' in this connexion is somewhat questionable.

Fig. 1. X-ray powder diagrams of sialic acid isolated from (a) ganglioside, (b) sheep submaxillary mucin (Guinier camera. Cu Kα-radiation).

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Fusarium nivale has a decisive effect upon the overwintering of rye in snow-covered fields. Generally speaking plant breeders have more and more begun to take the view that certain fungi often have a greater effect on the winter hardiness of winter cereals than frost. The possible anti-fungal factors in different over-wintering plants are therefore of great interest both from the biochemical and the plant breeding point of view. The first results of investigations on this line, in our laboratory, are briefly presented in this paper.

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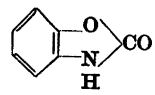
to separate lipids and other substances insoluble in water. On a one-dimensional paper chromatogram (solvent: *iso*propanol-ammonia (25 %)-water 8:1:1) the substance travelled near the solvent front. In zones where a strong auxin effect could be shown no anti-Fusarium effect was to be found in the concentrations used. Determinations with pure β-indole-acetic acid and 3-indoleacetonitrile showed that in concentrations in which they appear in plants they have no anti-Fusarium effect.

The extract was dissolved in *iso*propanol-water (80 : 20) and the insoluble part separated by centrifugation. The anti-Fusarium factor was separated from other substances in the extract in a cellulose powder column (9 × 50 cm) using as solvent *iso*propanol-water. When chlorophyll started to come out of the column 14 fractions of 200 ml each were taken. An anti-Fusarium activity could be found in fractions 2 and 3. These fractions were evaporated into dryness *in vacuo* and thoroughly shaken (glass beads added) with water. The water phase was separated. When evaporating the water solution crystals were formed. These crystals dissolved easily in hot water slightly in cold water. After three crystallizations the substance melted at 140° C. (Found: C 61.69; H 3.76; N 10.25 (Kjeldahl digestion with $KMnO_4$[1]). Calc. for $C_7H_5O_2N$: C 62.20; H 3.73; N 10.36.)

On the basis of its empirical formula and chemical and physical characteristics (*e. g.* aromatic ring, no ninhydrin reaction, no carboxyl group and the absorption spectrum in the ultra violet with a strong maximum at 270 mμ) it appeared possible that the compound might be 2(3)-benzoxazolinone. This substance was synthesized from *o*-aminophenol and urea according to the method of Baywater *et al.*[2] M. p. of the synthetic substance 141° C, mixed m. p. with the substance isolated by us 140° C. M. p. of the acetyl derivate of the synthetic substance 91° C, of the isolated substance 92° C, mixed m. p. 92° C. The UV spectra of isolated and synthetic benzoxazolinone were identical. Also on paper chromatograms both substances behaved identically in different solvent systems. The anti-Fusarium factor in rye seedlings was thus shown to be 2(3)-benzoxazolinone. As far as we are aware benzoxazolinone had not hitherto been found in Nature.



In our experiments on an agar medium the benzoxazolinone isolated by us inhibited the growth of *Fusarium nivale* completely in 0.05 % and weakly in 0.01 % solution. The amount of benzoxazolinone we could isolate in a crystalline form from fresh rye seedlings was 0.01 %. The actual content of the substance is, however, obviously higher. 2(3)-benzoxazolinone also inhibits the growth of clover-rot fungus *(Sclerotinia trifoliorum)* in the same concentrations as the growth of *Fusarium nivale*[3]. Red clover contains some factor which inhibits the growth of *Sclerotinia* and *Fusarium*[3]. The inhibitory substance in clover is being investigated.

Investigations on the content of benzoxazolinone in different varieties of rye and wheat are in progress. We are also investigating the mechanism of formation of this substance during germination. In our opinion the elucidation of the structures of natural anti-factors of different fungi causing plant diseases and destroying crops is of considerable importance in connection with plant breeding.

The authors wish to thank Professor V. A. Pesola, Director of the Division of Plant Breeding, State Agricultural Experiment Station, Jokioinen, for rye seeds, Professor E. A. Jamalainen, Director of the Division of Plant Disease, State Agricultural Experiment Station, Tikkurila, for the culture of *Fusarium nivale*, Professor O. Pohjakallio, University of Helsinki, for the culture of *Sclerotinia trifoliorum*. Professor E. R. H. Jones, the University of Manchester, England, for a gift of 3-indoleacetonitrile, and Mr. O. Pohjanheimo, Division of Plant Breeding, Jokioinen, for a valuable discussion with the senior author of this paper.

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Distribution of the 7α-Hydroxylating Activity in Rat Liver Homogenate Bile Acids and Steroids 35

SUNE BERGSTRÖM and URS GLOOR

Department of Physiological Chemistry, University of Lund, Lund, Sweden

The formation of cholic acid from desoxycholic acid by 7α-hydroxylation has been observed in the rat *in vivo* as well as in liver slices and homogenates [1-4]. This reaction was further studied with fractionated homogenates [4]. It was found, that a considerable part of the hydroxylating capacity was present in the particle free supernatant (centrifugation for 60 min, 100 000 × g).

The conclusions reached in this publication and the statement referring to this in a review article [5] have, however, to be corrected and modified in certain respects. When taurodesoxycholic acid is incubated with particle-free supernatant we have found that the yield of more polar products with the approximate mobility of taurocholic acid varies with the ATP * preparation used. Without added ATP no reaction products are observed (Table 1).

When Papst crystalline ATP (Pabst Lab. Milwaukee 3, Wisc.) is added, the yield is low but the product is practically pure taurocholic acid. With impure preparations of ATP (ATP-Ba-salt, Schwarz Lab. Inc., Mount Vernon, N. Y. Lot Nr ATB 5301; crude Barium salt from muscle) a large increase in the formation of polar compounds with approximately the same mobility of the filter paper in the system used (Butanol / 3 % acetic acid [6]) is found; see Table 1. With a slight modification in the conditions, the taurocholic acid can be clearly distinguished from the other metabolite.

As our chemical identifications accidently were done on runs with pure ATP yielding taurocholic acid we overlooked the presence of other products formed in the presence of impure ATP preparations.

The curve in Fig. 1 in paper [4] as well as Tables 1 and 3 thus represent taurocholic acid + unknown metabolites as these experiments were done with Schwarz ATP.

However, in the presence of microsomes (= particles spun down between 15 min, 20 000 × g and 60 min, 100 000 × g) plus supernatant, the product without or with pure ATP yielded essentially pure taurocholic acid, whereas again impure ATP led to an additional formation of the other metabolites.

Mitochondria or microsomes alone or with added ATP do not yield any taurocholic acid nor any other metabolite from taurodesoxycholic acid.

It therefore is clear that the microsomes and the particle free supernatant in com-

* Abbrevation: ATP = Adenosine triphosphate.

Table 1. Conversion of 150 μg taurodesoxycholic acid-24-^{14}C in rat liver homogenate fractions. 1ml incubation medium [4] corresponding to 200 mg liver fresh weight. Incubation 2 hrs, 37°, pH = 7.4, air. Amounts in μg.

	Microsomes + supernatant (centrifuged homogenate, upper layer after 15 min, 20 000 × g)			Supernatant (upper layer 60 min, 100 000 × g)		
	without ATP	with pure ATP	with impure ATP	without ATP	with pure ATP	with impure ATP
Taurocholic acid formed	68	97	97	<1	15	3
Unknown, more polar subst. formed	<1	7	43	<1	2	78

bination are responsible for the main part of the 7α-hydroxylating capacity. Only a minor part is found in the supernatant alone in the presence of pure ATP.

Impure ATP preparations contain a factor causing the formation of relatively large amounts of other polar products from taurodesoxycholic acid *in vitro*. In *in vivo* experiment with bile fistula rats practically the only product formed from desoxycholic acid is cholic acid.

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Infrared Absorption Spectra of some Salts of DL-2-Phosphoglyceric Acid

ANDREAS ROSENBERG and BO G. MALMSTRÖM

Institute of Biochemistry, University of Uppsala, Sweden

In a previous study [1], the strength of interaction between D,L-2-phosphoglyceric acid (PGA), the substrate of the enzyme enolase, and metal ions activating this enzyme was determined. Analogously to the concept of metal-ion activation of peptidases developed by Smith and his associates [2], it was suggested that the metal ion furnishes one of the points of interaction between enzyme and substrate. Thus, knowledge of the nature of the complexes between PGA and activating ions is important in interpreting the mechanism of the enzymic reaction. Studies by Rosenberg [3] have shown that the formation of metal chelates of amino acids results in characteristic changes in infrared absorption which can be correlated with the type of bond involved. In attempts to obtain similiar information about some metal-ion complexes of PGA, their infrared absorption spectra have been recorded.

PGA was synthesized according to Kiessling [4] and purified as described by Warburg and Christian [5]. To form a metal complex, a 0.02 M solution of the neutral Na^+ salt of PGA, containing an equimolar amount of metal chloride, was allowed to stand for about 1 h at room temperature. Then, absolute ethanol was added until a turbidity formed. (With the pure Na^+ salt, 64 vol. % ethanol was required, while the Mn^{++}, Zn^{++} and Ni^{++} complexes precipitated at concentrations of 13, 11 and 17 %, respectively.) After the addition of ethanol, the sample was placed in a refrigerator (4°) for 12 h. The precipitate formed was centrifuged off and dried in a vacuum dessicator over P_2O_5. Analysis showed the metal and acid to be present in a 1:1 ratio, as in solution [1]. A Perkin-Elmer model 21 recording spectrophotometer equipped with a NaCl prism was used for the absorption measurements. The substances were examined as pressed KBr discs prepared according to Schiedt and Reinwein [6].

Some typical results are shown in Figs. 1–3, which give the spectra of the Na^+, Zn^{++} and Ni^{++} salts of PGA. The absorption spectrum of each substance was recorded twice; the second time the sample was cooled to −170° by the use of a cell described by Rosenberg [3]. As seen in the figures, the use of the low temperature resulted in considerably better resolution as compared to room temperature.

The infrared absorption spectra of PGA and its salts are dominated by bands arising from the absorption by two highly polar groups, namely carboxyl and phosphate. The carboxyl group is characterized by its antisymmetrical vibration (ν|C(=O)O). It has been established by numerous investigations summarized by Bellamy [7] that this vibration of the ionized carboxyl group has a frequency of 1 600–1 590 cm^{-1} (about 6.3 μ). The Na^+ salt of PGA can be regarded as a normal salt, and it exhibits, as expected, a broad and intense band at approximately 1 590 cm^{-1}. If the carboxyl group were to form a bond of more covalent character with Zn^{++} and Ni^{++}, a shift of this band to higher wave num-

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PGA was synthesized according to Kiessling [4] and purified as described by Warburg and Christian [5]. To form a metal complex, a 0.02 M solution of the neutral Na^+ salt of PGA, containing an equimolar amount of metal chloride, was allowed to stand for about 1 h at room temperature. Then, absolute ethanol was added until a turbidity formed. (With the pure Na^+ salt, 64 vol. % ethanol was required, while the Mn^{++}, Zn^{++} and Ni^{++} complexes precipitated at concentrations of 13, 11 and 17 %, respectively.) After the addition of ethanol, the sample was placed in a refrigerator (4°) for 12 h. The precipitate formed was centrifuged off and dried in a vacuum dessicator over P_2O_5. Analysis showed the metal and acid to be present in a 1:1 ratio, as in solution [1]. A Perkin-Elmer model 21 recording spectrophotometer equipped with a NaCl prism was used for the absorption measurements. The substances were examined as pressed KBr discs prepared according to Schiedt and Reinwein [6].

Some typical results are shown in Figs. 1–3, which give the spectra of the Na^+, Zn^{++} and Ni^{++} salts of PGA. The absorption spectrum of each substance was recorded twice; the second time the sample was cooled to −170° by the use of a cell described by Rosenberg [3]. As seen in the figures, the use of the low temperature resulted in considerably better resolution as compared to room temperature.

The infrared absorption spectra of PGA and its salts are dominated by bands arising from the absorption by two highly polar groups, namely carboxyl and phosphate. The carboxyl group is characterized by its antisymmetrical vibration (ν|C(=O)O). It has been established by numerous investigations summarized by Bellamy [7] that this vibration of the ionized carboxyl group has a frequency of 1 600–1 590 cm^{-1} (about 6.3 μ). The Na^+ salt of PGA can be regarded as a normal salt, and it exhibits, as expected, a broad and intense band at approximately 1 590 cm^{-1}. If the carboxyl group were to form a bond of more covalent character with Zn^{++} and Ni^{++}, a shift of this band to higher wave num-

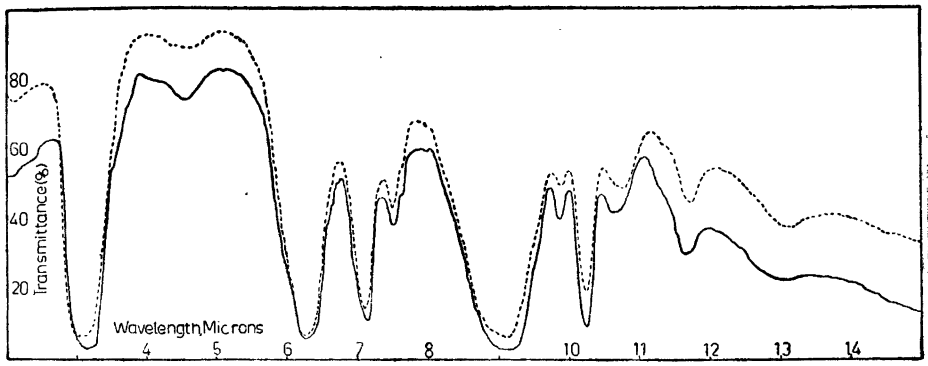

Fig. 1. The infrared absorption spectrum of the Na^+ salt of PGA. In this and the following figures, the dashed curve was recorded at room temperature and the solid curve at — 170°.

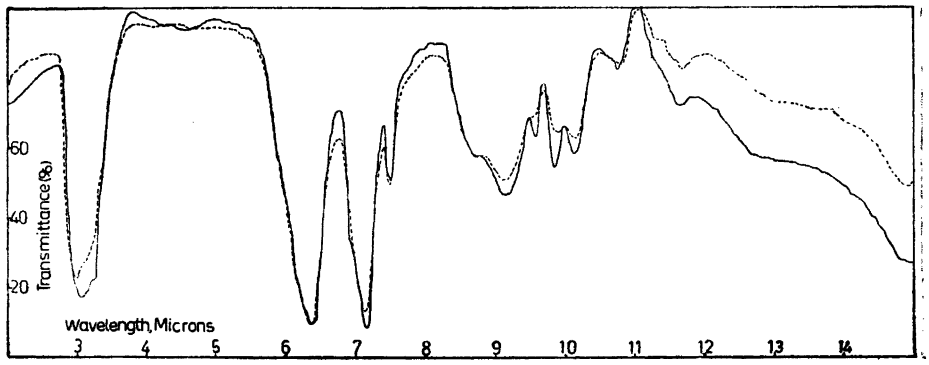

Fig. 2. The infrared absorption spectrum of the Zn^{++} complex of PGA.

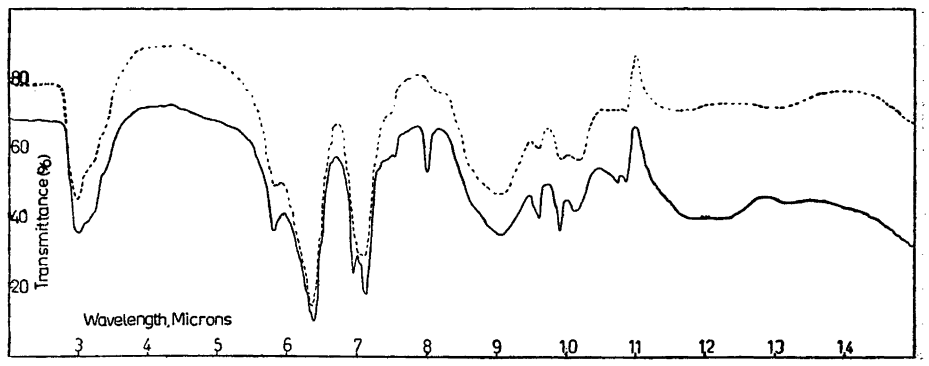

Fig. 3. The infrared absorption spectrum of the Ni^{++} complex of PGA.

bers would be expected. Such a behavior has been observed by Rosenberg [3] for different metal chelates of amino acids, where, for example, the carboxyl frequency at 1 590 cm^{-1} of the Na^+ salt showed a shift of 29 cm^{-1} in Cu^{++} chelates and of 55 cm^{-1} in Pt^{++} compounds. The carboxyl frequency in the Zn^{++} and Ni^{++} complexes of PGA shows no such trend. On the contrary, a small shift towards lower wave numbers can be observed. If this shift has any significance of its own is difficult to decide as the position of the band for the Na^+ salt is very approximative owing to its broadness; but it is clear, anyhow, that the carboxyl group shows no evidence for covalent bond formation with Zn^{++} and Ni^{++}.

The absorption due to the other dominant group, the ionized phosphate, exhibits remarkable changes when Na^+ is substituted by Ni^{++} or Zn^{++}. Unfortunately, there is very little information available on the infrared absorption of ionized phosphate groups [7], which makes it difficult to interpret the changes in the spectra. The intense absorption band between 1 150 and 1 050 cm^{-1} (8.7—9.5 μ) (probably due to the ionized group and P—O—C stretchings) loses much of its intensity in the Zn^{++} and Ni^{++} complexes. In the Zn^{++} complex, the absorption peak shows a tendency to split into two bands and at the same time small changes in the position of the maximum absorption can be detected. The absorption bands in the 1 050—950 cm^{-1} (9.5—10.5 μ) region, also characteristic of the phosphate group, show a more complex picture in the Zn^{++} and Ni^{++} compounds compared to the Na^+ salt.

Zn^{++} activates enolase while the Ni^{++} enzyme is inactive [8]. As an explanation for this, it has been suggested, on the basis of ultraviolet absorption measurements, that the Ni^{++} complex with the substrate is of a different type than the complexes with the activating ions [8]. The infrared absorption data reported here show that such a difference, if it exists, cannot be the amount of covalent character of the interaction with the carboxyl group, since this is completely ionic in both complexes. On the other hand, distinct differences exist between the phosphate absorption bands of the Zn^{++} and Ni^{++} complexes, but unfortunately these cannot be properly evaluated at present.

This investigation is part of a program on the infrared absorption of compounds of biochemical interest supported by the *Nobel Fund*.

1. Malmström, B. G. *Arch. Biochem. and Biophys.* **49** (1954) 335.
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5. Warburg, O. and Christian, W. *Biochem. Z.* **310** (1942) 385.
6. Schiedt, V. and Reinwein, H. *Z. Naturforsch.* **7b** (1952) 270.
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Free Amino Acids in Pollen

ARTTURI I. VIRTANEN and SIGRID KARI

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

In the summer 1954 we studied the composition of the free amino acid fraction in the pollen especially of wind-pollinated plants. The method used was that generally employed in this laboratory for the identification of free amino acids in green plants, *i. e.* homogenisation of the plant material, extraction with 70 % alcohol, separation of amino acids with Amberlite IR-120, elution with 1 N ammonia, and subjection of the evaporated extract to two-dimensional paper chromatography with butanol-acetic acid and phenol-ammonia.

The wind-pollinated plants investigated are: *Alnus incana, Betula alba, Corylus avellana, Quercus robur, Pinus silvestris, Populus balsamifera, Populus tremula, Secale cereale, Salix caprea.* For the identification of the spots of hydroxyproline and citrulline a special colour reaction was used: the spot of hydroxyproline turns red[1], and that of citrulline yellow [2], when treated with isatin + *p*-dimethylaminobenzaldehyde (PDB). The amounts of the amino acids were roughly estimated on the basis of the intensity of the spots.

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The wind-pollinated plants investigated are: *Alnus incana, Betula alba, Corylus avellana, Quercus robur, Pinus silvestris, Populus balsamifera, Populus tremula, Secale cereale, Salix caprea.* For the identification of the spots of hydroxyproline and citrulline a special colour reaction was used: the spot of hydroxyproline turns red[1], and that of citrulline yellow [2], when treated with isatin + *p*-dimethylaminobenzaldehyde (PDB). The amounts of the amino acids were roughly estimated on the basis of the intensity of the spots.

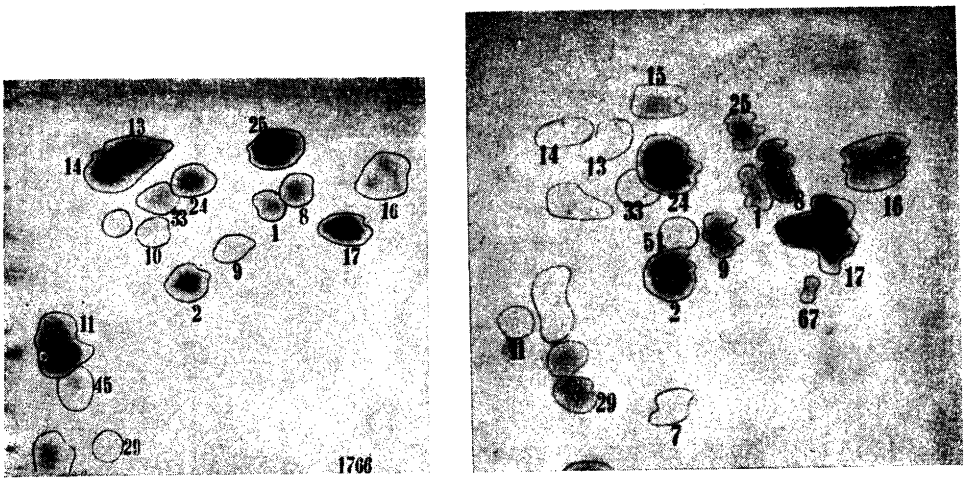


Fig. 1. Free amino acids of Betula alba. *Twodimensional paper chromatogram (phenol-NH_3 and butanol-acetic acid). A: pollen (210 µg N), B: leaves (182 µg N). 1 = gly, 2 = ala, 3 = val, 7 = tyr, 8 = ser, 9 = threo, 10 = OH-pro, 11 = pro, 13 = hist, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 19 = glutathione, 24 = glu-NH_2, 25 = asp-NH_2, 29 = γ-aminobut, 33 = citr, 45 = ethanolamine, 51 = homoser, 60 = pipecolic acid, 67 = α-aminoadipic acid.*

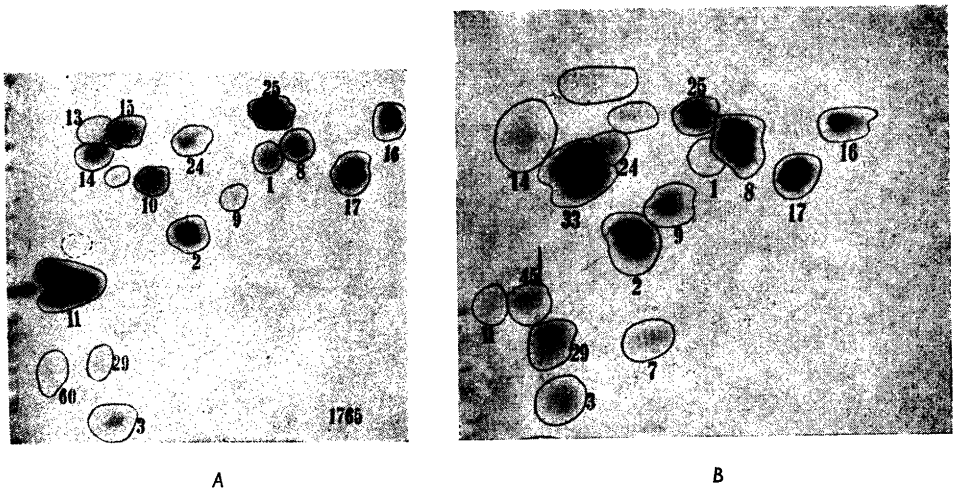
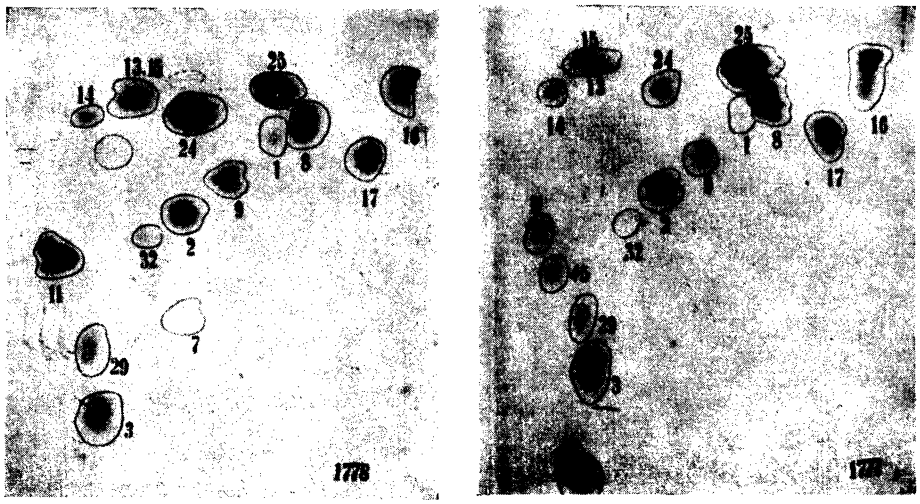


Fig. 2. Free amino acids of Corylus avellana. *A: pollen (210 µg N), B: leaves (200 µg N).*



A B

Fig. 3. Free amino acids of Populus balsamifera. *A: pollen (210 μg N), B: leaves (196 μg N). 32 = β – al.*

As examples of our results chromatograms of the free amino acids in *Betula*, *Corylus*, and *Populus balsamifera* are presented in Figs. 1–3. From these chromatograms some of the typical differences between the free amino acids in the pollen and in the leaves of the plants appear.

The most important results of our investigations are:

1. There is much more of free proline in the pollen than in the green parts of plants. Also the absolute amount of free proline is mostly high in the pollen. Because the dry matter content of pollen is very high, about 90 %, it is probable that the high proline content in pollen depends on this. Since the water content of the green parts of plants is usually high, 75–90 %, the formation of pollen is accompanied by a sharp decrease in the water content. Kemble and Macpherson [3] recently discovered that during the wilting of perennial rye grass the proline content increases so sharply that a considerable amount of proline must be synthesized during wilting. Glutamic acid is probably the precursor of proline because the enzymatic reaction: L-glutamic acid ⇌ L-proline is established[4].

2. Free hydroxyproline, the occurrence of which is comparatively rare in plants, is to be found in the pollen of four of the investigated plants *(Alnus, Corylus, Betula, Salix)*, but not in the green parts of any of these plants. It is possible that hydroxyproline is formed *via* oxidation of proline as has been found to be the case in the animal organism [5].

3. In addition to the high content of proline an imino acid containing a 6-membered ring, pipecolic acid (piperidine-2-carboxylic acid) is found in the pollen of most of the investigated plants *(Alnus, Corylus, Quercus, Betula, Salix, Secale)*, but not in the green parts. The biosynthesis of this acid from lysine is established [6]. The reaction is reversible. It is not known if drying promotes this synthesis.

The occurrence of proline in much higher amounts in the pollen than in the leaves of wind-pollinated plants is an interesting phenomenon. The formation of proline from glutamic acid leads to neutralization of this acidic amino acid and also to enrichment of carbon and nitrogen in the molecule. In the pollen of some plants pipecolic acid is formed from lysine (*cf.* above) which causes neutralization of this basic amino acid. Because the formation of proline is, however, many times greater than that of pipecolic acid, ringformation in its entirety lowers the acidity of the free amino acid fraction. From these imino acids the corresponding acidic and basic amino acids, respectively, may be formed

again during fructification. These circumstances can be of physiological significance.

4. Citrulline which is present in noticeable amounts in the leaves of *Alnus* and in large amounts in the leaves of *Corylus* could not be found in pollen. In other investigated plants citrulline does not occur, except in *Betula* where a small amount of it was found both in leaves and in pollen.

5. The amount of free basic amino acids is much higher in the pollen than in the leaves of many plants, as in *Betula*, *Populus tremula*, *Quercus*, and *Pinus*. A noticeable increase in amides (asparagine or glutamine or both) is also often found in pollen. When comparing the free amino acid fraction in pollen and in the green parts of plants it seems that the decrease in acidity of this fraction (*via* ringformation, formation of amides, increase in basic amino acids) is characteristic for the pollen of wind-pollinated plants.

Of the pollen of insect-pollinated plants only the free amino acids in the pollen of *Amaryllis* and *Lilium tigrinum* have been investigated. In these plants no typical differences between the amino acids in the pollen and in the green parts of the plants could be found. Because of the scantiness of the material no general conclusions can, however, be drawn about the free amino acids in the pollen of insect-pollinated plants.

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5. Stetten, M. R. and Schoenheimer, R. *J. Biol. Chem.* **153** (1944) 113; Stetten, M. R. *Ibid.* **189** (1951) 504.
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Dissociation Constants in the Yeast Alcohol Dehydrogenase System, calculated from overall Reaction Velocities

AGNAR P. NYGAARD* and HUGO THEORELL

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

As shown recently [1], the overall data obtained for yeast alcohol dehydrogenase ** (ADH) are consistent with the formation of a ternary complex where all equilibria are adjusted rapidly except for the transformation of the ternary complex (step 5).

For this scheme, Alberty [2] has shown that K_3, the dissociation constant of the third step, is identical with the Michaelis constant of DPN, K_4 the Michaelis constant of EtOH etc. Furthermore, the new kinetic constant introduced by Alberty [2],

$$K_{\text{EtOH·DPN}} = K_1 \cdot K_3 = K_2 \cdot K_4 \text{ and}$$
$$K_{\text{AldD·PNH}} = K_6 \cdot K_8 = K_7 \cdot K_9.$$

From these relationships the dissociation constants of ADH · EtOH ($= K_1$), of ADH · DPN ($= K_2$) etc. can be calculated. In Table 1 are shown the data obtained using the kinetic constants of the previous work [1]. Although the conditions of

* Fellow, Norwegian Research Council.

** The following abbreviations are used: ADH, yeast alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; EtOH, ethyl alcohol; Ald, acetaldehyde.

1. ADH + EtOH ⇌ ADH · EtOH
2. ADH + DPN ⇌ ADH · DPN
3. ADH · EtOH + DPN ⇌ ADH · EtOH·DPN
4. ADH · DPN + EtOH ⇌ ADH·EtOH·DPN
5. ADH · EtOH · DPN ⇌ ADH · Ald · DPNH
6. ADH · Ald · DPNH ⇌ ADH · Ald + DPNH
7. ADH · Ald · DPNH ⇌ ADH · DPNH + Ald
8. ADH · Ald ⇌ ADH + Ald
9. ADH · DPNH ⇌ ADH + DPNH

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Table 1. Dissociation constants obtained from overall reaction velocities compared with those obtained by Hayes and Velick [3] by direct measurements.

	From overall reaction velocities		By direct measurements [3]
	23° C pH 6.0	23° C pH 7.15	0—4° C pH 7.8
K_1, ADH · EtOH	0.10	0.074	—
K_2, ADH · DPN	1.6×10^{-4}	2.3×10^{-4}	2.6×10^{-4}
K_8, ADH · Ald	0.54×10^{-4}	1.4×10^{-4}	1.8×10^{-4}
K_9, ADH · DPNH	0.43×10^{-5}	1.0×10^{-5}	1.3×10^{-5}

the experiments were somewhat different, there is a fair agreement with the dissociation constants which Hayes and Velick [3] obtained by their ultracentrifugal method. The results add evidence to the proposed reaction mechanism for yeast alcohol dehydrogenase.

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Esters and Amides of 2,6-Dimethyl- and 2,4,6-Trimethylphenylcarbamic Acid

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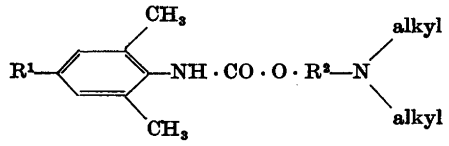
Central Laboratories, AB Astra, Södertälje, Sweden

A number of esters and amides of 2,6-dimethyl- and 2,4,6-trimethylphenylcarbamic acids with basic substituents have been prepared and tested for local anesthetic activity. The esters were active but rather toxic. The amides were devoid of anesthetic properties.

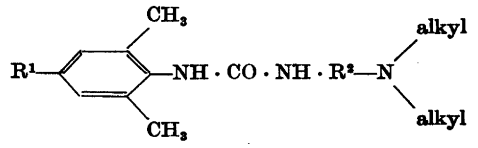
Esters of arylcarbamic acids possessing basic substituents have been reported to be active local anesthetics [1–7]. As it was of interest to study compounds of this class with the same substituents in the aromatic nucleus as Xylocaine, we prepared a series of basically substituted esters of 2,6-dimethyl- and 2,4,6-trimethylphenylcarbamic acids

(I; R^1= H or CH_3; R^2= —$CH_2 \cdot CH_2$— or —$CH(CH_3) \cdot CH_2$—)

and our experiments are reported in this paper. In addition some amides of the carbamic acids with basic substituents were prepared (II; R^1 = H or CH_3; R^2 = —$CH_2 \cdot CH_2$— or —$CH_2 \cdot CH_2 \cdot CH_2$—)



I



II

The new compounds were easily obtained by the reaction of 2,6-dimethyl- and 2,4,6-trimethylphenyl*iso*cyanate with the appropriate alcohol or amine. The *iso*cyanates used as starting materials were prepared from 2,6-xylidine or mesidine and phosgene. They could also be obtained by distillation of the appropriate ethyl carbamate with phosphorus pentoxide.

The amino esters possessed local anesthetic properties of about the same order of magnitude as Xylocaine when tested on rabbit cornea. However, they all were considerably more toxic.

Some of the least toxic compounds were tested subcutaneously on man and compared with Xylocaine. They were found to have shorter duration.

The amides with basic substituents were devoid of anesthetic properties.

EXPERIMENTAL

2,6-Dimethylphenylisocyanate. (*a*) A mixture of 2,6-xylidine (121.8 g, 1.0 mole) and pyridine (253.1 g, 3.2 moles) was added with ice-cooling to a solution of phosgene (118.7 g, 1.2 moles) in toluene (850 ml). A vigorous reaction commenced. The reaction mixture was kept at 0° for 2 hours and then at 25° for 3 hours. The thick mixture was then treated with 2 N hydrochloric acid (2.4 l). The toluene layer was separated, the aqueous layer extracted with toluene (2 × 200 ml) and the combined toluene solutions were dried over calcium chloride. The solvent was evaporated *in vacuo* and the residue distilled giving a colourless oil (99.4 g, 68 %), b.p. 87–89°/12 mm; n_D^{20} 1.5360. The product was rather irritating to the skin and the eyes. (Found: C 73.7; H 6.29; N. 9.35. C_9H_9NO requires C 73.5; H 6.16; N 9.52).

(*b*) Ethyl 2,6-dimethylphenylcarbamate (5.8 g, 0.03 mole) was thoroughly mixed with phosphorus pentoxide (8.5 g, 0.06 mole) and heated under reflux *in vacuo* (10 mm) at 100° for 10 minutes. The product was then distilled giving a colourless oil (3.7 g, 84 %), b.p. 84–85°/10 mm; n_D^{20} 1.5357.

Ethyl carbamates prepared from the products prepared according to (*a*) and (*b*) had the same m.p. (81–82°) undepressed on admixture.

2,4,6-Trimethylphenylisocyanate. (*a*) This compound was prepared in 52 % yield from mesidine and phosgene according to the procedure described under (*a*) above. The product was distilled *in vacuo* giving an oil of b.p. 100–102°/10 mm, which soon solidified to white crystals of m.p. 44–45°. (Found: C 74.1; H 7.01; N 8.51. $C_{10}H_{11}NO$ requires C 74.5; H 6.88; N 8.69).

(*b*) The same product was prepared in 64 % yield by distillation of ethyl 2,4,6-trimethylphenylcarbamate with phosphorus pentoxide as described under (*b*) above.

2,4,6-Trimethylphenyl*iso*cyanate has been prepared by Eisenberg [8] according to procedure (*b*). Eisenberg gives b.p. 218–220° at normal pressure.

Ethyl 2,6-dimethylphenylcarbamate. (*a*) Ethyl chloroformate (32.6 g, 0.3 mole) was added dropwise to a solution of 2,6-xylidine (94.5 g, 0.78 mole) in benzene (150 ml). After the vigorous reaction had subsided the mixture was kept at room temperature for 30 minutes and was then heated to the boiling point. After cooling the precipitated xylidine hydrochloride was removed by filtration and the benzene solution was extracted with N hydrochloric acid and dried over sodium sulphate. The solvent was evaporated and the white crystalline residue (54.5 g, 94 %) of m.p. 76–78° was crystallised from light petroleum giving a product of m.p. 82–83°. (Found: C 67.8; H 7.97; N 7.34. $C_{11}H_{15}NO_2$ requires C 68.4; H 7.82; N 7.25).

(*b*) 2,6-Dimethylphenyl*iso*cyanate (0.44 g) was refluxed in ethanol (5 ml) for 15 minutes. Evaporation of the solvent afforded white crystals (0.52 g, 90 %), which were recrystallised from light petroleum, m.p. 81–82°, undepressed on admixture with the compound prepared by method (*a*).

Ethyl 2,4,6-trimethylphenylcarbamate. (*a*) This compound was obtained in 81 % yield from mesidine and ethyl chloroformate as described above. The product was crystallised from light petroleum, m.p. 89–90°. (Found: C 69.8; H 8.34; N 6.69. $C_{12}H_{17}NO_2$ requires C 69.5; H 8.27; N 6.76).

(*b*) Reaction of 2,4,6-trimethylphenyl*iso*cyanate with ethanol afforded the same compound in 93 % yield, m.p. 89–90°, alone or in admixture with the product from (*a*). The synthesis of this compound has also been described by Eisenberg [8]. However, the m.p. recorded by Eisenberg is 61–62°.

β-Chloroethyl 2,6-dimethylphenylcarbamate. A solution of 2,6-dimethylphenyl*iso*cyanate (18.5 g) and ethylene chlorohydrin (15.0 g) in toluene (50 ml) was refluxed for 2 hours. On cooling white crystals (12.5 g) of m.p. 79–81° separated. Concentration of the mother liquor yielded further crystals (4.6 g) of the same m.p. The combined products (17.1 g, 60 %) were recrystallised from toluene, m.p. 80–81°. (Found: C 58.3; H 6.39; Cl 15.7. $C_{11}H_{14}ClNO_2$ requires C 58.0; H 6.20; Cl 15.6).

β-Chloroethyl 2,4,6-trimethylphenylcarbamate. Prepared similarly in 71 % yield. M.p. 89—90° (from toluene). (Found: C 59.9; H 6.64; Cl 15.0. $C_{12}H_{16}ClNO_2$ requires C 59.6; H 6.67; Cl 14.7).

N-(2,6-Dimethylphenyl)-urea. 2,6-Dimethylphenyl*iso*cyanate (3.7 g) was dissolved in toluene (15 ml) and a slow stream of gaseous ammonia was passed through the mixture with ice-cooling. White crystals began to separate almost instantaneously. After 2 hours the precipitate was collected (3.6 g, 88 %) and recrystallised from methanol, m.p. above 300°. (Found: C 66.1; H 7.23; N 17.2. $C_9H_{12}N_2O$ requires C 65.8; H 7.37; N 17.1).

N-Diethyl-N¹-(2,6-dimethylphenyl)-urea. A solution of 2,6-dimethylphenyl*iso*cyanate (3.7 g, 0.25 mole) and diethylamine (3.7 g, 0.05 mole) in toluene (10 ml) was refluxed for 2 hours. The precipitate (5.2 g, 95 %) melted at 178—179.5°. Recrystallisation from light petroleum-ethanol (5 : 1) did not raise the melting point. (Found: C 71.7; H 8.65; N 12.6. $C_{13}H_{20}N_2O$ requires C 70.9; H 9.15; N 12.7).

N-Diethyl-N¹-(2,4,6-trimethylphenyl)-urea. This compound was prepared similarly in 71 % yield, m.p. 122—123° (from 30 % methanol). (Found: C 71.7; H 9.40; N 11.7. $C_{14}H_{22}N_2O$ requires C 71.75; H 9.46; N 12.0).

N-Ethyl-N¹-(2,6-dimethylphenyl)-urea. A solution of 2,6-dimethylphenyl*iso*cyanate (3.7 g) and ethylamine (5 ml) in toluene (20 ml) was kept at room temperature in a sealed bottle overnight. The precipitate (3.3 g, 69 %) was recrystallised from light petroleum-ethanol (2 : 1), m.p. 226—228° (decomp). (Found: C 68.8; H 8.55; N 14.4. $C_{11}H_{16}N_2O$ requires C 68.7; H 8.39; N 14.6).

N,N¹-Bis(2,6-dimethylphenyl)-urea. A solution of 2,6-dimethylphenyl*iso*cyanate (5.7 g) and 2,6-xylidine (4.7 g) in toluene (15 ml) was refluxed for 2 hours. The crystalline product (10.0 g, 97 %) was recrystallised from glacial acetic acid. It sublimated at 330°. (Found: C 75.5; H 7.46; N 10.5. $C_{17}H_{20}N_2O$ requires C 76.1; H 7.51; N 10.4).

N,N¹-Bis(2,6-dimethylphenylcarbamyl)-ethylenediamine. This compound was prepared similarly from 2,6-diphenyl*iso*cyanate (4.4 g) and ethylenediamine hydrate (1.2 g) in toluene (15 ml). The reaction product (5.1 g, 97 %) was purified by extraction with hot ethanol. It sublimated at about 310°. (Found: C 67.8; H 7.45; N 15.8. $C_{20}H_{26}N_4O_2$ requires C 67.8; H 7.39; N 15.8).

Basically substituted esters of 2,6-dimethyl- and 2,4,6-trimethylphenylcarbamic acids*

These compounds were all prepared in the same way. The appropriate *iso*cyanate (0.04 mole) was refluxed with a basic alcohol (0.06 mole) in thoroughly dried toluene (15 ml) for 2 hours. (Small amounts of moisture caused the formation of small quantities of high melting products, which could be identified as N,N¹-bis(2,6-dimethylphenyl)- and N,N¹-bis(2,4,6-trimethylphenyl)-urea respectively). The toluene solution was washed with water and then extracted with 2 N hydrochloric acid. The extract was made alkaline with sodium carbonate solution and the precipitated base, which usually soon solidified, was collected and recrystallised. When it was impossible to obtain the base in crystalline form it was converted to the hydrochloride or the oxalate by the addition of an ethereal solution of hydrogen chloride or oxalic acid to an ethereal solution of the base.

β-Diethylaminoethyl 2,6-dimethylphenylcarbamate oxalate. M.p. 112—114° (decomp.), from acetone : light petroleum; yield 66 %. (Found: C 57.7; H 7.41; N 7.76. $C_{17}H_{26}N_2O_6$ requires C 57.6; H 7.40; N 7.91).

β-Diethylaminoethyl 2,4,6-trimethylphenylcarbamate. M.p. 44—45° (from light petroleum); yield 81 %. (Found: C 69.1; H 9.39; N 10.3. $C_{16}H_{26}N_2O_2$ requires C 69.0; H 9.41; N 10.1).

β-Dimethylaminoisopropyl 2,6-dimethylphenylcarbamate oxalate. M.p. 156—157° (decomp., from acetone); yield 85 %. (Found: C 56.5; H 7.18; N 8.35. $C_{16}H_{24}N_2O_6$ requires C 56.5; H 7.11; N 8.23).

* After the present work had been completed, the preparation of some of these esters was reported in a series of Swedish patent applications from AB Bofors. However, no melting points or other characteristic data were given.

β-Dimethylaminoisopropyl 2,4,6-trimethylphenylcarbamate hydrochloride. M.p. 211—212° (decomp., from ethanol); yield 63 %. (Found: C 59.5; H 8.52; Cl 11.5. $C_{15}H_{25}ClN_2O_2$ requires C 59.9; H 8.38; Cl 11.8).

β-Morpholinoethyl 2,6-dimethylphenylcarbamate hydrochloride. M.p. 185—187° (decomp., from ethanol); yield 71 %. (Found: C 57.1; H 7.23; Cl 11.4. $C_{15}H_{23}ClN_2O_3$ requires C 57.2; H 7.36; Cl 11.3).

β-Piperidinoethyl 2,6-dimethylphenylcarbamate. M.p. 85—86° (from light petroleum); yield 71 %. (Found: C 69.7; H 8.66; N 10.3. $C_{16}H_{24}N_2O_2$ requires C 69.5; H 8.75; N 10.1).

β-Piperidinoethyl 2,4,6-trimethylphenylcarbamate. The base was isolated as its *oxalate*, m.p. 153—154° (decomp., from acetone); yield 74 %. (Found: C 59.7; H 7.22; N 7.58. $C_{19}H_{28}N_2O_6$ requires C 60.0; H 7.42; N 7.37). From the pure oxalate the *base* was obtained in crystalline form, m.p. 87—88° (from light petroleum). (Found: C 70.6; H 8.67; N 9.45. $C_{17}H_{26}N_2O_2$ requires C 70.3; H 9.03; N 9.65).

Basically substituted amides of 2,6-dimethyl- and 2,4,6-trimethylphenylcarbamic acids

The appropriate *iso*cyanate (0.025 mole) was refluxed with a dialkylaminoalkylamine (0.038 mole) in dry toluene (10 ml) for 2 hours. On cooling the base usually separated in crystalline form. Two compounds were isolated by precipitation of the toluene solution with ethereal oxalic acid.

γ-Diethylaminopropylamine and γ-piperidinopropylamine used as starting materials were prepared in *ca.* 90% yield by the reduction of β-diethylaminopropionitrile [9] and β-piperidinopropionitrile [9] with lithium aluminium hydride according to the general procedure described by Amundsen and Nelson [10].

N-(β-Diethylaminoethyl)-N¹-(2,6-dimethylphenyl)-urea. M.p. 105—106° (from methanol); yield 87 %. (Found: C 68.6; H 9.38; N 15.8. $C_{15}H_{25}N_3O$ requires C 68.4; H 9.57; N 15.95).

N-(β-Diethylaminoethyl)-N¹-(2,4,6-trimethylphenyl)-urea. M.p. 129—130° (from 70 % methanol); yield 80 %. (Found: C 69.4; H 9.48, N 15.2. $C_{16}H_{27}N_3O$ requires C 69.3; H 9.81; N 15.15).

N-(γ-Diethylaminopropyl)-N¹-(2,6-dimethylphenyl)-urea. The compound was isolated as the oxalate from which the crystalline base was obtained, m.p. 105—106° (from light petroleum); yield 65 %. (Found: C 68.9; H 9.63; N 15.1. $C_{16}H_{27}N_3O$ requires C 69.3; H 9.81; N 15.15).

N-(γ-Diethylaminopropyl)-N¹-(2,4,6-trimethylphenyl)-urea. The compound was isolated by means of oxalic acid. The base melted at 121—122° (from light petroleum); yield 65 %. (Found: C 69.5; H 9.85; N 14.6. $C_{17}H_{29}N_3O$ requires C 70.1; H 10.0; N 14.4).

N-(γ-Piperidinopropyl)-N¹-(2,6-dimethylphenyl)-urea. M.p. 127—128° (from light petroleum-ethanol 2 : 1); yield 71 %. (Found: C 70.3; H 9.20; N 14.8. $C_{17}H_{27}N_3O$ requires C 70.55; H 9.41; N 14.5).

N-(γ-Piperidinopropyl)-N¹-(2,4,6-trimethylphenyl)-urea. M.p. 168—170° (from ethanol-light petroleum 2 : 1); yield 97 %. (Found: C 70.9; H 9.31; N 13.9. $C_{18}H_{29}N_3O$ requires C 71.25; H 9.63; N 13.85).

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The Crystal Structure of Phosphoric Acid

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The crystal structure of anhydrous phosphoric acid, H_3PO_4, has been determined by X-ray methods. Three of the P—O bonds in the molecule, presumably the P—OH bonds, are found to be 1.57 Å, whereas the bond to the fourth oxygen is significantly shorter, 1.52 Å. The PO_4 group deviates slightly, but significantly from tetrahedral symmetry; it has within the limits of error trigonal symmetry. Two hydrogen bonds of length 2.53 Å connect the "keto" oxygen with "hydroxyl" oxygens, whereas the third bond between two "hydroxyl" oxygens is found to be 2.84 Å. Approximate positions derived for the hydrogens show that these atoms probably are ordered.

Phosphoric acid, phosphates, and phosphate esters are compounds of considerable interest from several points of view, and a detailed knowledge of their stereochemistry would appear to be desirable. A number of investigations of KH_2PO_4 have been carried out [1,2], but no other detailed structure analysis in this field appears to be reported in the literature. A preliminary note on the structure of phosphoric acid, H_3PO_4, based on Fourier projections along two axes was published in 1954 by the author [3]. Recently Smith, Brown and Lehr [4] have given an account of the structure on the basis of similar evidence. The present paper describes a more comprehensive analysis, the results of which differ in part considerably from those obtained by Smith, Brown and Lehr.

EXPERIMENTAL

Highly purified crystalline phosphoric acid from The British Drug Houses Ltd. was used for the investigation. All the work was carried out at room temperature. The crystals are very hygroscopic and have a low melting point (42° C). They were therefore studied and cut in dry CCl_4 and mounted in thinwalled glass capillaries. Partial melting and recrystallization in the capillaries was difficult to avoid. Weissenberg and oscillation photographs were taken about two crystallographic axes using filtered Cu$K\alpha$ radiation (λ = 1.542 Å). The intensities were estimated visually.

CRYSTAL DATA

The crystals are monoclinic, elongated along the b axis, usually with (001) dominating. Twinning on (001) was observed in one batch of crystals. The unit cell dimensions are: a = 5.78 Å, b = 4.84 Å, c = 11.65 Å, and β = 95.5°,

the values being accurate to within 0.5 %. The only systematic absences occur in the $h0l$ reflections for l odd and in the $0k0$ reflections for k odd, and the space group is $P2_1/c$. By flotation in mixtures of carbon tetrachloride and ethylene dibromide a density of about 2.0 g/cm³ was found, corresponding to four molecules in the unit cell. The calculated density is 2.00 g/cm³. The density of pure liquid phosphoric acid is 1.834 g/cm³ at 18° C.

In the literature the crystals are described as orthorhombic (Joly [5]), and a pseudo-orthorhombic unit cell may in fact be chosen.

THE STRUCTURE DETERMINATION

Projections of the structure in direction of the b- and a-axes were determined independently using Patterson, Fourier and difference methods.

The structure factors. In the b-axis projection all 83 reflections attainable with Cu$K\alpha$ radiation were recorded with measurable intensity. Two different crystals were used with cross-sections 0.12×0.15 mm and 0.20×0.25 mm, respectively. The agreement between the two sets of measurements was good and the mean value taken. The intensities were corrected for Lorentz and polarization effects, but not for absorption. In the a-axis projection a crystal of cross-section 0.14×0.25 mm was used and 66 out of 71 reflections were recorded.

The approximate structure. The positions of the phosphorus and some of the oxygen atoms were derived from Patterson projections, and after four Fourier refinements the electron density maps given in Fig. 1 were obtained. The resolution is not good as one oxygen is close to the phosphorus in both projections. Diffraction effects are also evident in the maps. The reliability index $R = (\Sigma|F_0 - F_c|) / \Sigma|F_0|$ was at this stage about 0.20. The coordinates

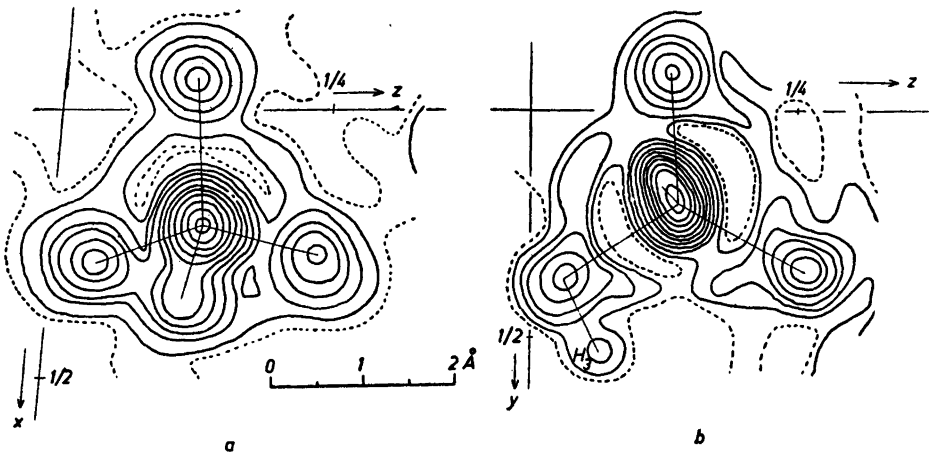

Fig. 1. Electron density projections on the (010) plane (a) and the (001) plane (b). Contours at 2, 4, 6, ... e · Å⁻², every second contour being omitted above 12 e · Å⁻². The broken line corresponds to 1 e · Å⁻². Calculated at 1/60 of c *and 1/30 of* a *and* b.

derived from the maps differed in part considerably from those finally obtained by (F_0—F_c) syntheses.

(F_0—F_c) refinement. The structure was further refined by successive (F_0—F_c) syntheses (Cochran [6]). The refinement was continued until at all atomic centres the synthesis and its slope were nearly zero, whereas only an approximate correction was made for anisotropy in the thermal vibrations.

In the *b*-projection eight successive difference syntheses were calculated. Corrections to the positional and vibrational parameters were derived partly from the difference maps, partly by plotting F_0/F_c against $\sin^2\Theta$. One adjustment of scale and temperature factors was also carried out by least squares calculation, in which 75 linear equations, one for each reliable reflection, were solved for four unknowns in groups corresponding to different directions in the reciprocal lattice. In the temperature factor exp ($-B \sin^2 \Theta/\lambda^2$) finally chosen B was 3.1 Å^2 for the oxygen atoms and 1.7—0.4 $\sin^2\varphi$ for the phosphorus atom, where φ is the angle between the normal of the reflecting plane and the direction of maximum vibration of the atom. This direction lies about 40° from *c* in the obtuse angle. In addition, the vibrations of the molecule as a whole appear to be slightly asymmetric with maximum vibration in a direction about 15° from *a* in the obtuse angle. This was corrected for by applying a factor exp ($-0.5 \sin^2\varphi' \cdot \sin^2\Theta/\lambda^2$). The final difference map is given in Fig. 2a. The highest peaks are probably due to hydrogen atoms, which were not included in F_c at this stage. Atomic coordinates are given in Table 1.

Table 1. Atomic coordinates as fractions of the corresponding cell edge.

Atom	*x*	*y*	*z*
P	211	202	1402
O_1	282	346	2538
O_2	328	910	1295
O_3	277	371	0330
O_4	942	157	1205

The *a*-projection was less completely refined. Five difference syntheses were calculated, but in the last map, which is given in Fig. 2b, there are still irregularities of considerable magnitude. Probably the intensity measurements were less accurate in this zone. A temperature factor with $B = 0.9—0.5 \sin^2\varphi''$ was applied to the oxygen atoms and $B = 0.9—1.4 \sin^2\varphi''$ to the phosphorus atom, where φ'' is the angle between *b* and the normal to the reflecting plane. It will be seen that the values of B are much lower in this projection. The temperature factors probably have little significance in terms of thermal motion of the atoms; rather, they represent also other effects such as absorption and variation in spot shape.

The atomic scattering curves given by Viervoll and Øgrim [7] were used in all the calculations. When the seven reflections suffering from extinction (see below) are not included, the reliability index R is 0.08 for the *b* projection and 0.13 for the *a* projection. In Table 5 observed and calculated structure factors are given (hydrogen contribution included).

The hydrogen atoms. The standard deviation in electron density is about 0.25 e $\cdot$Å^{-2} for the ($h0l$) zone and 0.6 e $\cdot$Å^{-2} for the ($0kl$) zone. Only the highest

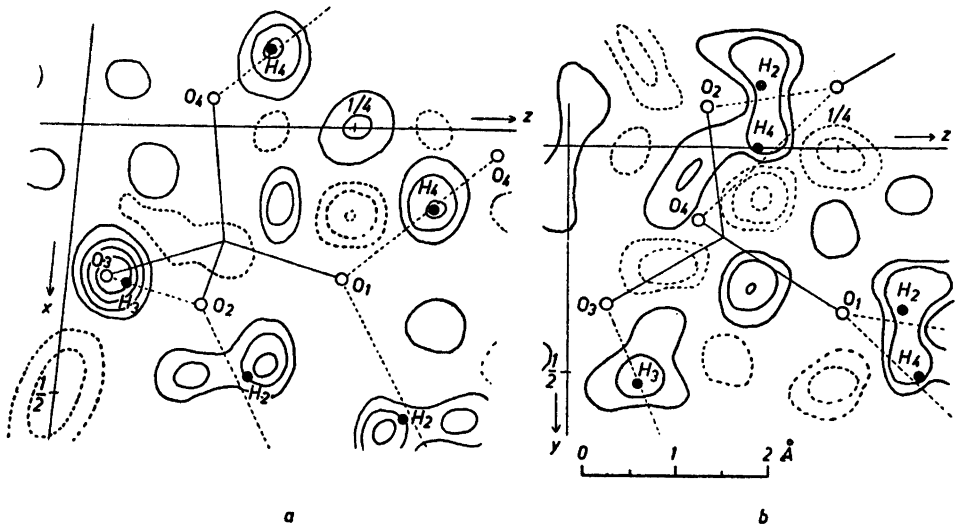

Fig. 2. Difference-syntheses on the (010) plane (a) and the (100) plane (b). In (a) the contour interval is 0.2 e · Å^{-2}, in (b) 0.5 e · Å^{-2}. Hydrogen contribution not included in F_c. Negative contours are broken and zero contour omitted. Hydrogen bonds are broken. The filled circles show the positions assigned to the hydrogen atoms.

peaks in the *b* projection (Fig. 2a) are therefore likely to be significant. They can reasonably be interpreted as indicating the positions of the three hydrogens, and show that these atoms probably are associated with oxygen atoms O_2, O_3 and O_4. There are no significant peaks near O_1. The hydrogen atom H_4 gives a well-defined peak, whereas H_2 is associated with a broad area near $O_2 \cdot H_3$ lies almost directly above O_3 and its height depends therefore on the choice of temperature factor for O_3. In the *a* projection (Fig. 2b), where the peaks are not significant, H_3 is well resolved, whereas H_2 and H_4 are poorly defined. H_3 also appears as a separate peak on the electron density map (Fig. 1b). The peak positions are very sensitive to changes in atomic coordinates and temperature factors, but it is believed that the assignment of the hydrogens is qualitatively correct. The following coordinates, which obviously are inaccurate, were derived: H_2 (0.46, —0.15, 0.18); H_3 (0.30, 0.54, 0.062); H_4 (0.15, 0.50, 0.33). The coordinates of H_3 and the x, z coordinates of H_4 were found directly from the maps. The others were deduced by taking the position of the hydrogen bonds into account. When the hydrogen contribution to the structure factors was included R dropped by about 0.01 to 0.07 and 0.12 for the two projections, respectively. Atoms H_2 and H_4 were given half weight as they take part in strong hydrogen bonds (Cochran [9], Bernal [10]).

Extinction. In Table 2 the observed uncorrected intensity (I_{obs}) and F_o and F_c of the seven strongest reflections are listed. It will be seen that the F_c values in all cases are much to high. The effect is thought to be due mainly to extinction. It presented a serious difficulty in the structure determination, making the calculation of scale and temperature

factors uncertain and preventing an experimental determination of the hydrogen contribution to these reflections. The reflections were not included in the calculation of the difference maps and their calculated values were used in deriving the electron density maps.

Table 2. The strongest reflections.

Reflection	I_{obs}	F_o	F_c	0.11 $F_o\sqrt{I_{obs}}$
$\bar{1}02$	352	8.2	17.6	16.9
012	320	8.6	14.2	16.9
102	304	8.0	17.0	15.3
200	248	8.8	18.3	15.3
013	214	7.7	12.8	12.4
004	175	7.2	10.8	10.5
302	125	8.3	10.8	10.2

Assuming the F_c values to be nearly correct, it was found empirically that the extinction error could be roughly corrected for by multiplying F_o by a factor $k\sqrt{I_{obs}}$ where k is a constant. The values of k varied from 0.10 to 0.13 and the mean value, 0.11, was used to derive the last column in Table 2. According to this the structure factors of the strongest reflections are approximately proportional to I_{obs} rather than to $\sqrt{I_{obs}}$ in the present case.

DESCRIPTION AND DISCUSSION OF THE STRUCTURE

Accuracy of the structure. The standard deviations of the atomic coordinates were estimated by applying the formula given by Cruickshank [8]. The following values were found: $\sigma(x) = \sigma(z) = 0.008$ Å, $\sigma(y) = 0.024$ Å for the oxygen atoms; $\sigma(x) = \sigma(z) = 0.002$ Å, $\sigma(y) = 0.005$ Å for the phosphorus atom. An experimental check on the accuracy is provided by the two independent determinations of the z coordinates. They differed by 0.005 Å for the phosphorus atom and by 0.004 Å, 0.023 Å, 0.012 Å and 0.010 Å for the oxygen atoms O_1, O_2, O_3 and O_4, respectively. These values give a standard deviation of 0.009 Å in the z coordinates of the oxygen atoms, about the same as found by Cruickshank's formula. From these results the standard deviations in the P—O bond lengths given in Table 3 were computed. The standard deviation of O—P—O bond angles were calculated to be approximately 0.7°. The standard deviation of electron density was found to be about 0.25 e · Å^{-2} in the b projection and 0.6 e · Å^{-2} in the a projection.

The molecular structure. From the coordinates in Table 1 the distances and angles given in Tables 3 and 4 and Fig. 3 were calculated. The bond P—O_1 is found to be 1.52 Å, considerably shorter than the other three P—O bonds, which are all close to 1.57 Å. The difference is significant. The bond lengths found by Smith, Brown and Lehr are also given in Table 3. As there appears to be hydrogen atoms attached only to O_2, O_3 and O_4, O_1 is the "keto" oxygen, in accordance with the shortness of the bond P—O_1.

The bond angles show small, but significant deviations from the tetrahedral angle. Those involving the bond P—O_1 are greater than 109.5°, their mean value being 112°, whereas the angles between two P—(OH) bonds all are near 106°. Correspondingly, the distances from the "keto" oxygen to the "hydroxyl" oxygens (2.56—2.59 Å) are somewhat greater than the distances

Table 3. Bond lengths.

Bond	Length (Å)	Standard deviation	Values from Ref.[4]
$P-O_1$	1.517	0.013	1.57
$P-O_2$	1.577	0.024	1.59
$P-O_3$	1.570	0.013	1.55
$P-O_4$	1.568	0.008	1.52

Table 4. Bond angles and O—O distances.

	Angle		Length (Å)
O_1-P-O_2	112.8°	O_1-O_2	2.59
O_1-P-O_3	112.2°	O_1-O_3	2.57
O_1-P-O_4	110.9°	O_1-O_4	2.56
O_2-P-O_3	105.3°	O_2-O_3	2.50
O_2-P-O_4	106.8°	O_2-O_4	2.52
O_3-P-O_4	105.3°	O_3-O_3	2.50

between "hydroxyl" oxygens (2.50—2.52 Å). The PO_4 group thus deviates slightly from tetrahedral symmetry, but has within the limits of error trigonal symmetry with the shortest bond as trigonal axis.

Smith, Brown and Lehr [4] found an irregularly distorted tetrahedron with one O—O distance as short as 2.34 Å.

It was found from the maps in Figs. 1a and 2a that the number of electrons in the phosphorus peak is 15.2 and in the oxygen peaks near 8.0. It is difficult to estimate the error because of partial overlap of atoms, uncertainties in the areas assigned to the atoms, and the extinction effect. The values indicate, however, that transfer of electrons, if any, only takes place to a small extent.

The values given by Pauling [11] for the length of single- and double-bonds P—O are 1.76 Å and 1.55 Å, respectively. Thus a very large amount of double-bond character is indicated for the three long P—O bonds of 1.57 Å. It is, however, less pronounced than for the shortest bond, which is only 1.52 Å. This bond is presumably a nearly pure double-bond, and it is possible that the

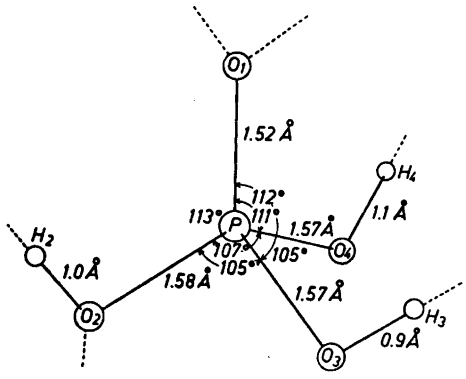

Fig. 3. The molecular structure of H_3PO_4. The broken lines indicate hydrogen bonds.

Table 5. Observed and calculated structure factors. The values given are one quarter of the absolute values. F_o's suffering from extinction in brackets.

hkl	F_o	F_c	*hkl*	F_o	F_c
002	2.9	— 2.6	400	6.3	6.4
004	(7.2)	—10.8	402	4.7	4.3
006	2.3	1.9	404	3.1	— 3.2
008	10.3	11.7	406	4.2	— 3.6
0 0 10	7.0	— 7.0	408	3.8	3.8
0 0 12	4.2	— 3.9	4 0 10	1.1	— 1.0
0 0 14	1.8	2.1			
			$\bar{4}02$	5.7	— 5.3
100	3.7	3.2	$\bar{4}04$	3.0	2.6
102	(8.0)	—17.0	$\bar{4}06$	5.7	5.9
104	5.9	— 6.3	$\bar{4}08$	1.4	1.2
106	5.9	6.2	$\bar{4}$ 0 10	4.8	— 4.8
108	5.8	— 5.9	$\bar{4}$ 0 12	1.9	2.1
1 0 10	5.2	— 5.1			
1 0 12	2.6	2.6	500	0.9	1.3
1 0 14	2.3	2.0	502	0.9	— 0.6
			504	4.5	— 4.6
$\bar{1}02$	(8.2)	17.6	506	1.9	2.0
$\bar{1}04$	5.4	— 5.2	508	1.0	— 0.3
$\bar{1}06$	4.3	— 4.7			
$\bar{1}08$	9.7	9.9	$\bar{5}02$	1.4	— 1.0
$\bar{1}$ 0 10	5.2	5.1	$\bar{5}04$	5.1	— 5.1
$\bar{1}$ 0 12	4.6	— 4.6	$\bar{5}06$	4.7	4.3
$\bar{1}$ 0 14	0.7	— 0.5	$\bar{5}08$	3.1	3.2
			$\bar{5}$ 0 10	1.9	— 2.0
200	(8.8)	— 18.3			
202	5.5	5.8	600	1.2	— 1.1
204	2.7	2.7	602	2.7	— 1.1
206	3.0	— 2.5	604	1.6	1.7
208	6.3	— 6.4	606	2.7	2.7
2 0 10	6.3	6.7			
2 0 12	3.5	3.6	$\bar{6}02$	2.5	2.7
			$\bar{6}04$	3.2	— 2.8
$\bar{2}02$	2.7	— 2.7	$\bar{6}06$	2.7	— 2.9
$\bar{2}04$	3.8	— 3.2	$\bar{6}08$	1.7	1.5
$\bar{2}06$	2.0	— 1.5			
$\bar{2}08$	4.3	— 4.4	700	2.8	— 2.6
$\bar{2}$ 0 10	5.7	5.6	$\bar{7}02$	0.9	0.1
$\bar{2}$ 0 12	1.1	— 1.0	$\bar{7}02$	1.1	0.9
$\bar{2}$ 0 14	1.4	— 1.5	$\bar{7}04$	4.1	4.1
300	4.3	3.9	011	4.6	4.1
302	(8.3)	10.8	012	(8.6)	—14.2
304	3.4	3.1	013	(7.7)	—12.8
306	7.5	— 7.6	014	1.5	— 0.8
308	4.8	4.1	015	6.1	— 5.9
3 0 10	5.2	5.3	016	6.2	6.2
3 0 12	1.8	— 1.7	017	6.5	5.7
			018	7.3	— 7.2
$\bar{3}02$	7.7	— 8.8	019	4.0	3.2
$\bar{3}04$	2.6	2.4	0 1 10	6.3	— 6.0
$\bar{3}06$	1.0	1.2	0 1 11	3.8	— 3.6
$\bar{3}08$	4.1	— 3.9	0 1 12	4.5	4.1
$\bar{3}$ 0 10	2.8	— 2.5	0 1 13	< 1.0	0.7
3 0 12	1.7	1.9	0 1 14	2.2	2.4

hkl	F_o	F_c	*hkl*	F_o	F_c
020	7.9	− 9.0	040	4.7	− 4.7
021	3.2	2.2	041	5.3	5.5
022	4.3	3.2	042	2.1	− 0.9
023	4.6	− 4.6	043	5.9	6.6
024	4.4	4.7	044	2.6	− 2.2
025	8.5	0.2	045	7.5	− 9.0
026	2.7	− 2.0	046	2.4	1.9
027	2.9	1.6	047	2.1	− 1.5
028	5.8	− 5.6	048	1.7	− 1.6
029	< 1.5	0.7	049	5.5	5.6
0 2 10	5.4	5.0	0 4 10	< 0.7	0.3
0 2 11	< 1.5	− 0.1	0 4 11	0.8	1.0
0 2 12	1.8	1.8			
0 2 13	4.8	4.6	051	3.8	3.5
			052	2.9	2.3
031	4.7	− 4.4	053	3.7	− 2.7
032	6.9	6.7	054	1.8	1.4
033	7.9	9.4	055	< 0.9	− 0.6
034	4.4	− 3.5	056	1.0	− 0.8
035	5.0	4.8	057	4.1	3.8
036	6.4	− 7.2	058	1.0	1.1
037	5.9	− 6.3			
038	3.0	2.0	060	2.5	2.3
039	2.7	− 2.4	061	3.5	− 3.7
0 3 10	3.1	2.9	062	1.0	− 1.1
0 3 11	5.6	5.2	063	2.3	− 2.0
0 3 12	4.4	− 4.6			

value for a double-bond P—O should be revised slightly downwards from 1.55 Å. The values observed for the bond angles are in accordance with the different degree of double-bond character of the P—O bonds. One would expect a smaller bond angle between two bonds with partial single-bond character than between one such bond and a pure double-bond.

In the phosphorus oxyhalides a P—O distance of 1.55 ± 0.03 Å and bond angles of 106° (Cl—P—Cl) and 112° (Cl—P—O) are found [12]. The angles are thus the same as in the present structure. In crystals of KH_2PO_4 all P—O bonds are reported to be equal, 1.56 Å, at room temperature, whereas below the Curie point, where ordering of the hydrogens is assumed, two sets of bonds of lengths 1.53 Å and 1.58 Å are found (Frazer and Pepinsky [2]). These values are close to those found in the present investigation (1.52 Å and 1.57 Å), where the hydrogens also appear to be ordered.

The hydrogen bonds. Only three intermolecular oxygen-oxygen separations are shorter than 3.0 Å, and as they all involve a hydrogen atom, they correspond to hydrogen bonds. Two of these are very short, 2.53 Å, connecting the "keto" oxygen O_1 with "hydroxyl" oxygens O_2 and O_4 in different neighbouring molecules. They firmly connect molecules related by screw axes into sheets parallel to the (001) plane. The third hydrogen bond is longer, 2.84 Å, forming a link between "hydroxyl" oxygens O_3 and O_2 in molecules related by *b* translation, thus further connecting the molecules within the sheets. There are no hydrogen bonds between the sheets. The two short bonds have the same length

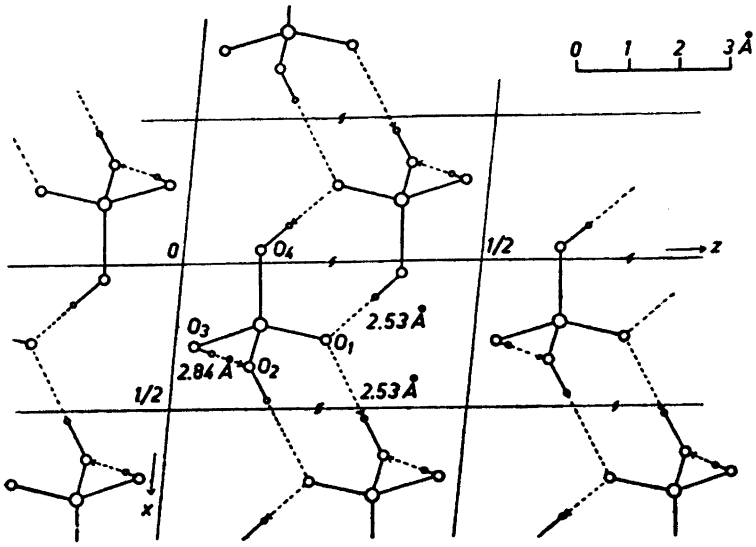

Fig. 4. The b *projection of the structure. Arrows indicate that the hydrogen bonds (broken) terminate at atoms related to those shown by* b *translation.*

as the hydrogen bonds found in KH_2PO_4 at room temperature (2.53 Å). The hydrogen bonds are shown in Fig. 4. (In Fig. 1 in the author's preliminary note [3] the hydrogen bond from O_3 is erronously drawn.)

The formation of the strong hydrogen bonds does not seem to influence the state of the molecule to any great extent. The P—(OH) bonds are found to be nearly equal, and the two strong hydrogen bonds have the same length.

According to Bernal [10] the electronic charge around the proton will decrease from about 0.6 electrons for the weaker "hydroxyl" bonds of length 2.75—2.85 Å to about 0.3 for the stronger bonds of length 2.65—2.4 Å. He also assumes that the O—H bond may lengthen from 0.85 Å to about 1.10 Å as the hydrogen bonds increase in strength. The charge distribution and O—H distances indicated by the difference maps in Fig. 2 appear to be in general agreement with this view, although little significance can be attached to the values derived. In the weak bond between two hydroxyls (2.84 Å) the O_3—H_3 distance is found to be 0.89 Å and the charge 0.45 el., whereas in the short bond involving H_4 the O_4—H_4 distance is 1.1 Å and the charge only 0.3 el. The hydrogen atom of the other strong bond (H_2) is very poorly defined (see above), but the coordinates assumed correspond to an O—H distance of 1.0 Å. The charge on H_2 is 0.25 el. The number of electrons associated with the hydrogens were computed from the (*h*0*l*) difference map (Fig. 2a) after adding to the map the calculated hydrogen contribution to the reflections suffering from extinction.

To locate the hydrogen atoms more precisely better intensity measurements are required, as well as a more careful refinement of both projections of the structure.

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Studies on the Extraction of Metal Complexes

XXI. The Complex Formation of Thorium with Tropolone

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A study on chloroform extraction of the Y(III), La(III), Th(IV) and U(VI) tropolonates showed that Th and U may be separated very well from the trivalent ions.

Calculations on the distribution curve for Th gave a mean complexity constant of 8.14 for the formation of the Th tropolonate. This value is higher than for the acetylacetonate, but lower than for the oxinate.

Tropolone has a structure such that one might suppose that it would form extractable metal complexes. This supposition is supported by some statements by Cook and Loudon [1] and by Doering and Knox [2] concerning the solubility of the Cu(II) and Fe(III) tropolonates in chloroform. A study was therefore undertaken to discover if Th(IV), U(VI) and trivalent metals like Y(III) and La(III) could be separated by chloroform extraction using tropolone as the complexing agent.

Apart from the interest in solvent extraction separations, it is possible to determine from the data on Th the complex formation between Th^{4+} and the tropolonate ion (A^-). Previously such investigations have been published by our group for the ligand ions acetylacetonate [3], oxinate [4] and cupferrate [4]. Studies on the complex formation of some bivalent metals with tropolone have been carried out by Bryant, Fernelius and Douglas [5].

EXPERIMENTAL

The experimental technique has been described in several of the papers in this series (*cf. e. g.* Ref.[4]) and all experiments were again carried out at 25° C. The tropolone, which was supplied by Dr. Hans Fernholz, was dissolved in alcohol-free, water-saturated chloroform. The ionic strength in the aqueous phase was kept constant at 0.1 M using 0.1 M $HClO_4$, $NaClO_4$ and NaOH, and the initial volumes of the two phases were 15 ml. The distribution of Th, Y and La was measured radiometrically using $^{234}Th(UX_1)$, ^{140}La and ^{90}Y as tracers. The distribution of U, however, was determined by spectrophotometric

Table 1. Distribution of U(VI) between chloroform and 0.1 M perchlorate solutions at 25° C as a function of the tropolonate ion concentration in the aqueous phase. $[U]'_{aq} = 10^{-4}$ *M.*

$[HA]'_{org}$ M	A_{400}	log q [a]	−log [H+]	−log [A⁻]
0.05	0.452	+0.33	1.03	8.68
0.05	0.488	+0.45	1.15	8.56
0.05	0.561	+0.74	1.24	8.47
0.05	0.607	+1.04	1.53	8.18
0.05	0.655	+1.91	2.00	7.71
0.05	0.659	+2.21	2.31	7.40
0.025	0.112	−0.69	1.02	8.99
0.025	0.171	−0.46	1.12	8.89
0.02	0.132	−0.60	1.00	9.11
0.02	0.189	−0.40	1.24	8.87
0.02	0.342	+0.03	1.52	8.59
0.02	0.649	+1.67	2.09	8.02

a) $q = A_{400}/(0.663 - A_{400})$

Table 2. Distribution of Th-^{234}Th between chloroform and 0.1 M perchlorate solutions at 25° C as a function of the tropolonate ion concentration in the aqueous phase.

$[HA]'_{org}$ M	−log $[Th]'_{aq}$	I_{org} cpm	I_{aq} cpm	log q	−log [H+]	− log [A⁻]
1	2	3	4	5	6	7
0.1	5.70	6 429	1 095	+0.80	1.07	8.34
0.1	5.88	13 426	672	+1.33	1.29	8.12
0.1	5.70	6 905	366	+1.30	1.34	8.07
0.1	5.88	10 952	163.0	+1.86	1.64	7.77
0.1	5.70	7 381	114.3	+1.84	1.67	7.74
0.1	5.88	13 817	79.6	+2.27	2.19	7.22
0.1	5.88	12 940	12.2	+3.05	2.55	6.86
0.1	5.88	12 751	6.2	+3.34	2.77	6.64
0.1	5.88	11 135	7.4	+3.21	5.82	3.59
0.1	5.88	11 040	4.1	+3.46	5.96	3.45
0.1	5.88	11 004	5.2	+3.35	6.43	2.98
0.1	5.88	12 680	3.4	+3.60	6.45	2.96
0.1	6.18	7 911	−0.8	—	6.86	2.56
0.1	5.70	7 487	7.1	+3.05	6.98	2.45
0.1	5.70	7 317	33.2	+2.37	7.73	1.76
0.1	5.88	12 582	412	+1.51	8.74	1.17
0.05	5.70	6 152	7 275	−0.05	1.05	8.66
0.05	5.70	9 577	3 373	+0.48	1.38	8.33
0.05	5.70	11 726	1 105	+1.05	1.61	8.10
0.05	5.70	12 533	359	+1.57	1.89	7.82
0.05	5.88	9 510	115.6	+1.94	2.07	7.64
0.05	5.70	12 573	53.2	+2.40	2.16	7.55
0.05	5.88	9 649	105.8	+1.99	2.20	7.51
0.05	5.88	9 757	85.9	+2.08	2.23	7.48
0.05	5.88	9 492	6.8	+3.17	3.52	6.19
0.05	5.88	7 915	8.6	+2.99	4.47	5.24
0.05	5.88	8 058	3.8	+3.36	4.79	4.92

1	2	3	4	5	6	7
0.05	5.88	7 930	4.5	+3.27	6.37	3.34
0.05	5.88	8 038	12.3	+2.84	7.26	2.48
0.05	5.88	9 422	17.2	+2.77	7.30	2.44
0.05	5.88	9 311	71.8	+2.14	8.10	1.78
0.05	5.88	8 510	547	+1.22	10.33	1.30
0.02	5.88	1 446	10 539	−0.84	1.07	9.04
0.02	5.88	2 666	8 992	−0.50	1.34	8.77
0.02	5.88	5 767	4 985	+0.09	1.63	8.48
0.02	5.88	8 583	1 886	+0.69	1.96	8.15
0.02	5.70	11 755	1 683	+0.87	2.03	8.08
0.02	5.70	11 861	952	+1.12	2.32	7.79
0.02	5.70	12 978	249	+1.74	2.67	7.44
0.01	5.88	100.0	11 159	−2.02	1.04	9.37
0.01	5.88	361	10 849	−1.45	1.09	9.32
0.01	5.88	554	13 787	−1.37	1.29	9.12
0.01	5.88	2 128	9 904	−0.64	1.56	8.85
0.01	5.88	2 490	11 742	−0.65	1.60	8.81
0.01	5.88	6 700	7 067	+0.00	1.88	8.53
0.01	5.88	7 698	4 054	+0.31	2.03	8.38
0.01	5.88	9 331	2 108	+0.67	2.20	8.21
0.01	5.88	12 801	682	+1.30	2.48	7.93
0.01	5.88	11 599	18.9	+2.82	4.97	5.44
0.01	5.88	11 354	23.3	+2.71	7.58	2.89
0.005	6.18	37.3	11 223	−2.45	1.10	9.61
0.005	6.18	90.7	11 071	−2.06	1.37	9.34
0.005	6.18	337	10 783	−1.48	1.66	9.05
0.005	6.18	1 378	9 649	−0.82	1.95	8.76
0.005	6.18	4 623	6 121	−0.09	2.26	8.45
0.005	6.18	8 704	3 552	+0.42	2.54	8.17
0.002	6.18	11.2	9 449	−2.90	1.33	9.78
0.002	6.18	36.9	9 293	−2.37	1.69	9.42
0.002	6.18	135.0	9 184	−1.80	1.92	9.19
0.002	6.18	684	8 615	−1.07	2.20	8.91
0.002	6.18	2 110	7 339	−0.51	2.50	8.61
0.002	6.18	5 087	4 184	+0.12	2.88	8.23
0.001	6.18	21.5	8 885	−2.59	1.51	9.80
0.001	6.18	28.4	8 925	−2.47	1.81	9.50
0.001	6.18	212	7 992	−1.56	2.26	9.16
0.001	6.18	1 619	7 155	−0.62	2.70	8.72
0.001	6.18	5 274	3 380	+0.22	3.05	8.37

* Standard deviation: $\sqrt{I}/\sqrt{5}$ cpm.

measurements of the chloroform phases at 400 mμ using a Beckman DU spectrophotometer with 1 cm cells. With Y and La only a few experiments were made, as the solubilities of these tropolonates in chloroform are only about $27 \cdot 10^{-6}$ M and $5 \cdot 10^{-6}$ M.

The hydrogen ion concentration, $-\log[H^+]$, was measured by means of a Radiometer pHM3i potentiometer equipped with glass and calomel electrodes, which were checked against 0.1 M buffers of known $-\log[H^+]$. The free ligand ion concentration in the aqueous phase, $-\log[A^-]$, was calculated using the stoichiometric dissociation constant $10^{-6.71}$ and the partition coefficient $10^{1.70}$ given in Part XVIII [6].

In the Tables, $[HA]'_{org}$ refers to the initial concentration of tropolone in the chloroform phase, and $[U]'_{aq}$ and $[Th]'_{aq}$ refer to the initial total concentrations of U and Th

in the aqueous phase. I_{org} and I_{aq} in Table 2 are the measured radioactivities of equal volumes of the phases and the distribution ratio q is given by

$$q = \frac{I_{org}}{I_{aq}} \cdot (1.067 \pm 0.010)$$

The correction factor 1.067 is introduced because the ratio I_{org}/I_{aq} does not quite correspond to the ratio of Th concentrations due to differences in absorption and scattering of beta particles. The correction factor was calculated from the data for the Th-tropolone system with the method described in Part VI [4].

The partition coefficient of ThA_4 was determined from 16 values on the plateau of the distribution curve (see Fig. 2)

$$\lambda_4 = \frac{I_{org}}{I_{aq}} = \frac{190\,756}{140.2 \pm 5.3} \cdot (1.067 \pm 0.010) = 1\,450 \pm 70$$

$$\log\lambda_4 = 3.16 \pm 0.02$$

RESULTS

Fig. 1 shows that UO^{2+} and Th^{4+} may be separated very well from trivalent ions like Y^{3+} and La^{3+} by extraction with *e. g.* a 0.05 M chloroform solution of tropolone at pH 2. The sequence for Th^{4+}, Y^{3+} and La^{3+} is that expected from the ionic charges and radii (1.02, 0.92 and 1.14 Å). As with cupferron [7] the extraction of La^{3+} is poor, the solubility of La tropolonate being even less in chloroform than in the aqueous phase ($\lambda_3 < 1$).

The data for U(VI) are given in Table 1. The range of log q is too small for the calculation of complexity constants. However, if log q is plotted against $-\log[A^-]$ a straight line is obtained with a slope equal to 2. The equation for this line is (eqn. 7 in Part V[8])

$$\log q = \log \lambda_2 \varkappa_2 + 2 \log [A^-]$$

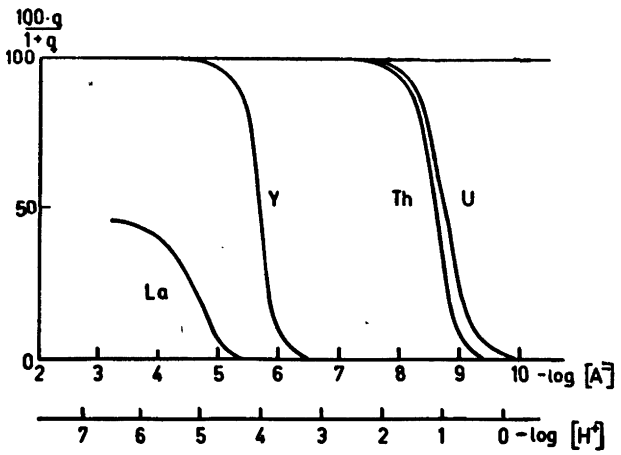


Fig. 1. The distributition of Y(III), La(III), Th(IV) and U(VI) between chloroform and 0.1 M perchlorate solutions as a function of the tropolonate ion concentration. The corresponding values of $-\log[H^+]$ are given for the case when an aqueous solution is equilibrated with an equal volume of a 0.05 M solution of tropolone in chloroform.

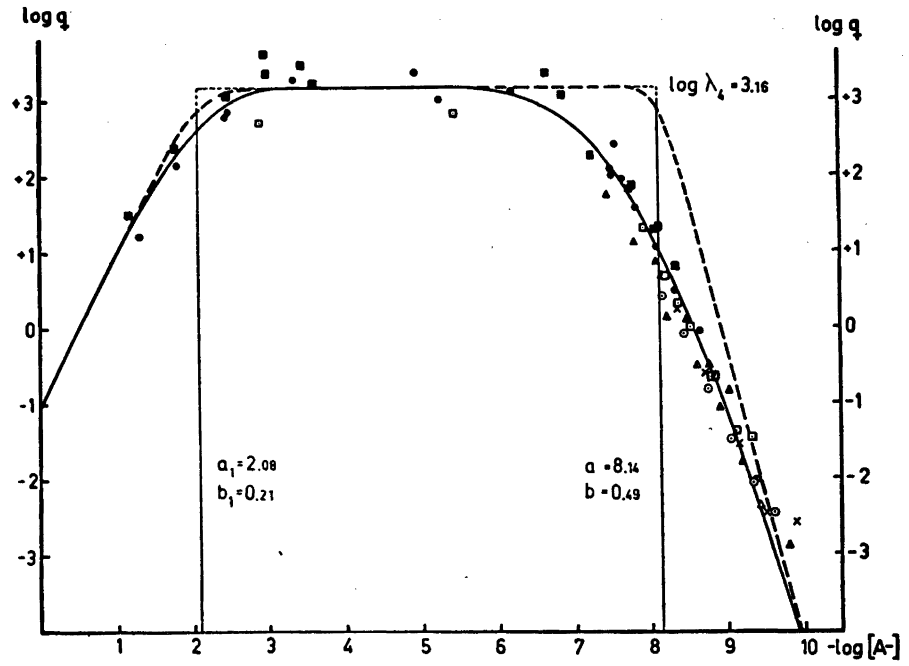


Fig. 2. The distribution ratio of Th as a function of the tropolonate ion concentration in the aqueous phase. To the right positive complexes predominate in the aqueous phase and to the left negative complexes seem to be formed. In the intermediate range the uncharged complex ThA_4 predominates (case III in Part V[8]). Initial concentration of tropolone in the chloroform phase: ■ 0.1 M; ● 0.05 M; ▲ 0.02 M; □ 0.01 M; ○ 0.005 M; △ 0.002 M; × 0.001 M.

where

$$\log \lambda_2 \varkappa_2 = 17.48$$

The data for thorium are given in Table 2, and are plotted in Fig. 2. The full curve in this figure is calculated with the two-parameter method described in Part V [8] (case III) using the following parameters:

$$a = 8.14$$
$$b = 0.49$$
$$\log \lambda_4 = 3.16$$
$$a_1 = 2.08$$
$$b_1 = 0.21$$

The parameter a expresses the mean complexity constant 10^a and the complexity product

$$\varkappa_4 = \frac{[ThA_4]}{[Th^{4+}][A^-]^4} = k_1 k_2 k_3 k_4 = 10^{4a} = 10^{32.56}$$

The parameter b expresses the mean spreading factor of the complexity constants k_n

$$k_n / k_{n+1} = 10^{2b} = 10^{0.98}$$

The complexity constants are defined as

$$k_n = \frac{[\mathrm{ThA}_n]}{[\mathrm{ThA}_{n-1}]\,[\mathrm{A}]} = 10^{a+b(5-2n)}$$

and hence

$$k_1 = 10^{9.61} \qquad k_3 = 10^{7.65}$$
$$k_2 = 10^{8.63} \qquad k_4 = 10^{6.67}$$

The distribution constant of Th tropolonate is

$$\lambda_4 = \frac{[\mathrm{ThA}_4]_{org}}{[\mathrm{ThA}_4]_{aq}} = 10^{3.16}$$

Furthermore, the results at high tropolonate ion concentrations indicate that negative complexes, ThA_5^- and ThA_6^{2-}, are also formed. These complexity constants are obtained from the parameters a_1 and b_1

$$k_5 = 10^{a_1+b_1} = 10^{2.29}$$
$$k_6 = 10^{a_1-b_1} = 10^{1.87}$$

The dashes represent the curve calculated assuming that the only Th complexes formed are ThA_4 and ThA_6^{2-}. Obviously this is not so.

DISCUSSION

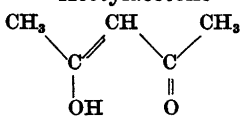
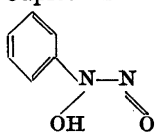
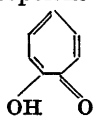
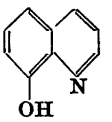
It has been shown by Bryant, Fernelius and Douglas [5] that the complex formation of Cu(II) with acetylacetone is weaker than with tropolone and this seems also to be the case for thorium.

Table 3 lists the results for thorium that have been published so far by our group. As new data on other reagents will be published shortly a general discussion of our results will be deferred. However, some properties, which seem to influence the complexity constants of Th^{4+} may be indicated briefly.

From Table 3 it is obvious that no general statements such as "five-membered chelate rings are more stable than six-membered rings" or "acid strength correlates with chelate stability" are sufficient to explain the behavior of these different agents with Th^{4+}.

No such correlation as the latter should be expected as hydrogen (on the basis of infrared measurements [9] and theoretical calculations [10]) appears to form one short covalent bond and one long ionic hydrogen bond with the two donor atoms in the chelate ring, while thorium would very likely form only ionic bonds. It seems as if there is a marked correlation between the infrared vibration frequency of the OH-bond and the calculated length of the H-bond except for cupferron. This may indicate that the chelate ring in cupferron is not planar due to a O—O repulsion. The distance in acetylacetone is especially

Table 3. Summary of data on some chelating agents.

	Acetylacetone	Cupferron	Tropolone	Oxine
Structure				
No. of atoms in chelate ring	6	5	5	5
O—O(N)distance (Å) in chelate ligand [a]	2.40	2.15	2.50	2.80
OH—O(N)distance [a] (Å)	1.40	1.45	1.90	2.25
Frequency (cm^{-1}) of infrared abs. max. for OH-band	2 650	3 360	3 100	3 430
Length (Å) of OH-bond from infrared data [b]	1.12	1.00	1.04	0.99
Dissociation constant of of HA, pk_a	8.81 [c]	4.16	6.71	9.66
Mean complexity constant of ThA_4, $\frac{1}{4}\log \varkappa_4$ $(= a)$	6.70 [d]	6.75	8.14	9.70
Mean spreading factor, $\log k_n - \log k_{n+1}$ $(= 2\ b)$	0.90 [d]	0.40	0.98	0.50
Partition coefficient of HA between chloroform and water, $\log k_d$	1.37 [e]	2.18	1.71	2.66
Partition coefficient of ThA_4 between chloroform and water, $\log \lambda_4$	2.57 [d]	2.79	3.16	2.39

a) Calculated for planar chelate rings from covalent bond radii and approximate bond angles.
b) Calculated from Badger's [11] rule $\omega^2 (r—0.335)^3 = k$, using $\omega = 3\ 620\ cm^{-1}$ and $r = 0.97$ Å for naphthol and phenol.
c) Reported by Rydberg [12]. The value for the pure enol form (calculated from the keto-enol equilibrium constant of 0.205 reported by Eidinoff [13]) is 8.05.
d) Calculated by the two-parameter method [8] from data given by Rydberg [3].
e) Reported in Part IX [12]. The value for the pure enol form (calculated from data by Eidinoff [13] and Meyer [14]) is 2.03.

favorable for a strong chelate binding of hydrogen. In the case of thorium it seems as if the long O—N distance in the oxinate ligand is more favorable for complex formation than the somewhat shorter O—O distance in tropolone. The much lower complexity constants for the acetylacetonate and cupferrate ligands may very well be explained by the fact that these ligands are not stabilized in one plane by an aromatic ring, but may rotate around a bond in the part of the ligand ion, which forms the chelate ring.

The author wishes to thank the head of FOA 1, Professor G. Ljunggren for his continuous interest in these metal extraction studies.

I am also very grateful to Mr. Ebbe Johansson for his valuable technical assistance.

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On the Isomerism of Hydroxyurea

VI. Paper-Partition Chromatography

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A paper-chromatographic technique for analytical separation and identification of the alleged isomers, melting at 72° C ("OU_{72}") and at 130—140° C ("OU_{140}") respectively, is described in detail. Samples of OU_{140}, prepared according to the usual ionic process (I) have been found to be contaminated with minor amounts of a closely related substance, provisionally named OU_X, the constitution of which is not yet known.

Heat-induced interconversion processes have been studied qualitatively in neutral, acid and alkaline aqueous solutions.

The irreversible isomerization $OU_{72} \rightarrow OU_{140}$, postulated by previous investigators, has been proved.

The discoverers of the lower-melting hydroxyurea (m. p. 72° C), Francesconi and Parrozzani, in their paper [1] stated that the substance, when heated in ethanolic solution, was easily converted to the previously known isomer, melting at 140° C. This was apparently inferred from the fact that an ethanolic solution, after having been boiled, produced a blue-violet colour with ferric chloride, similar in appearance to that produced by the isolated higher-melting hydroxyurea. The authors did not submit experimental documentation.

In 1907 Conduché [2], citing the Italian paper, added that in aqueous solution the lower-melting hydroxyurea was partly converted to the higher melting isomer and partly decomposed. He also reported that the inverse transformation of the higher-melting isomer into the lower-melting one had proved unsuccessful. Experimental evidence was lacking in this paper too.

The aim of the work, described in the present communication, was to supply direct proof — or disproof — that the process responsible for the vanishing of the lower-melting hydroxyurea (OU_{72}) in solution leads to the formation of the higher-melting hydroxyurea (OU_{140}). Since the paper-chromatographic technique under favourable conditions is an extremely sensitive analytical tool, it was also hoped to reveal a possible formation of other products and in general to obtain useful information on the nature of the conversion process. Such information was also desirable, because a planned kinetic investigation

by means of the polarographic method, developed in the preceding paper, would be profitable only if the process were relatively clear-cut. Finally there were still some doubts concerning the homogeneity of the isolated hydroxyurea, melting at 140° C (X-ray powder diagrams, *vide* Ref. 3; the difficulties in getting well-developed single crystals, Ref. 3 p. 486). Paper-chromatography should be well suited for settling this question.

In the search for a satisfactory spray detecting reagent 1 N ferric chloride was first tested. It is known to produce an intense blue-violet colour with OU_{140}, similar to that formed with hydroxamic acids, to which OU_{140} is probably closely related. The lower limit of sensitivity is approximately 5 μg in a spot 1 cm in diameter (Whatman No. 1 paper). However, OU_{72} gives but a faint red, quickly fading colouration in relatively high concentrations, and ferric chloride is therefore quite inadequate as a detecting reagent for this isomer.

A satisfactory universal spray reagent for both isomers is picryl chloride, originally recommended by Bremner [4] for the detection of hydroxylamine on paper chromatograms.

Upon spraying with a 1 % ethanolic solution of picryl chloride OU_{72} is revealed by a bright red colour, which gradually developes in the course of a minute or two, and which is further intensified by subsequent exposure of the dry chromatogram to ammonia vapour.

In contradistinction to OU_{72} the isomeric OU_{140} gives no colour reaction with picryl chloride alone. At high concentrations (>100 μg) the spot is discernible as a discoloured area on the yellow background. If, however, the dry chromatogram is subquently exposed to ammonia vapour, a bright orange, for higher concentrations a brown, colour is produced spontaneously. This colour is very similar to that produced by hydroxylamine salts but less dense for equal quantities of material in the spots.

The different behaviour of OU_{72} and OU_{140} toward picryl-chloride/ammonia is in itself of diagnostic value and permits safe differentiation. The lower limit of sensitivity is in either case approximately 1 μg in a spot, 1 cm in diameter. The most characteristic colours and best developed spots were obtained with 50 to 100 μg applied to the paper. The red colour produced by OU_{72} fades away in the course of some days, whereas the orange colour characteristic of OU_{140} is more stable. Presence of mineral acids in the paper inhibits the colour reaction. Care should therefore be taken to remove any trace of acid prior to spraying, when acidic solvents are being used for irrigation. With ethanol/hydrochloric acid it was sufficient to dry the chromatogram at room temperature overnight.

First of all the homogeneity of OU_{140} was investigated. The sample was prepared as previously reported [5] by the ionic process

$$HONH_3^+ + {}^-OCN \rightarrow CH_4O_2N_2 \qquad (I)$$

conducted at low temperature. The reaction mixture is known to contain $OU_{72} + OU_{140}$. The former was extracted from the freeze-dried mixture by means of anhydrous ether, then the latter was isolated by extraction with hot absolute ethanol, from which it crystallized upon cooling to room temperature. Fig. 1, No. 2 shows a typical paper-chromatogram of OU_{140}, deve-

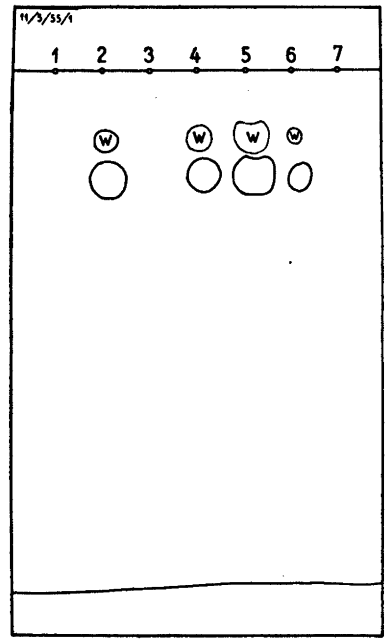


Fig. 1. Solvent isobutanol > water, 16 hours. Spray-reagent picryl chloride/ammonia. w weak, colour of all spots orange. 1. *hydroxylammonium chloride 50 µg;* 2. OU_{140} *100 µg;* 3. *nil,* 4. *freshly prepared reaction mixture from process I, see text, 10 µl;* 5. OU_{140} *100 µg, 6 months old sample;* 6. *as* 4., *5 µl solution;* 7 *as* 1.

loped with *iso*butanol, saturated with water. Two completely separated spots are formed, having the R_F-values *ca.* 0.20 and 0.14 respectively. The spots produced the same colour with picryl chloride/ammonia. The slow spot was always smaller and less densely coloured than the fast one. A duplicate chromatogram sprayed with ferric chloride revealed that only the fast spot responds to this reagent (blue-violet colour), occasionally the slow spot was just discernible as a very faint reddish colour on the dry chromatogram. This suggests that OU_{140} is responsible for the principle spot R_F 0.20 and in the following this spot will be denoted accordingly. The possible origin of the slow, subsidiary spot (for the sake of brevity denoted OU_X) will be discussed below.

In Fig. 1, No. 4 is reproduced a paper-chromatogram of a fresh reaction mixture from process (I). 5 millimoles of the reactants were separately dissolved in 25 ml of water, and the solutions mixed at room temperature. 10 µl of the solution, containing approximately 1 % hydroxyurea were applied to the paper. Two spots are developed, corresponding to OU_{140} and OU_X, whereas OU_{72} does not appear on this chromatogram. This is partly because it is formed by process (I) predominantly at low temperature, and partly because it is to a large extent converted to OU_{140} during the long period of development (16 h). More satisfactory chromatographic conditions for OU_{72} are described below.

It is obvious that the slow spot OU_X found in all our samples of OU_{140} cannot be due to a contamination with OU_{72}, since this substance responds in an entirely different manner to picryl chloride/ammonia. This conclusion was borne out by comparative paper-chromatography of isolated samples of OU_{140}

and OU_{72} on the same paper strip. These experiments showed that OU_{72} is distinctly different from either spot OU_{140} and OU_X also by flow rate (*cf.* Tables 1—3 and Fig. 2, No. 6). Furthermore, during ealier reported polarographic work[15], OU_{140}-samples have never been found to be contaminated with OU_{72}.

Since the hydroxylammonium ion and cyanate ion constitute the starting materials in the preparation of the hydroxyureas, it should be considered, whether OU_X be identical with hydroxylammonium cyanate; although this

Table 1. Paper-chromatography of hydroxyurea. Isobutanol > water, *25° C. 100 µg substance in initial spot 1 cm in diameter.*

Substance	R_F	Spray reagents		
		picryl chloride	picryl chloride /ammonia	ferric chloride
OU_X	0.14	no colour	orange	faint red
OU_{140}	0.22	no colour	orange	blue-violet
OU_{72}	0.27	weak red	red	faint red
Hydroxylamine		no colour	no colour	no colour

Table 2. Paper-chromatography of hydroxyurea. Ethanol-ammonia pH ca. 12—13; 25° C. 100 µg substance in initial spot 1 cm i diameter.

Substance	R_F	Spray reagents		
		picryl chloride	picryl chloride /ammonia	ferric chloride
OU_X	0.27	no colour	orange	faint red
OU_{140}	0.53	no colour	orange	blue-violet
OU_{72}	0.60	weak red	red	faint red
Hydroxylamine		no colour	no colour	no colour

Table 3. Paper chromatography of hydroxyurea. Ethanol-hydrochloric acid, pH ca. 0; 25° C. 100 µg substance in initial spot 1 cm in diameter.

Substance	R_F	Spray reagents		
		picryl chloride	picryl chloride /ammonia	ferric chloride
OU_{72}	0.43	weak red or yellow	red	faint red
Hydroxylamine	0.48	no colour	orange	no colour
OU_{140}	0.67	no colour	orange	blue-violet

substance has never been isolated in solid state, it might be present in samples of OU_{140} either as an impurity, due to incomplete reaction (I) or possibly existing in equilibrium with hydroxyurea in solution, analogous to the well-known equilibrium ammoniumcyanate ⇌ urea. This explanation was, however, disproved by the observation that hydroxylamine salts cannot be detected at all on paper chromatograms run with neutral and alkaline solvents in the concentrations applied in the experiments, *vide* Fig. 1, No. 1 and 7 and Fig. 2, No. 4, *cf.* Ref. 4. On chromatograms run with acidic solvents (Fig. 4, No. 3) the hydroxylammonium ion is easily recognizable, whereas the spot OU_X has vanished. This may also be taken as an indication of non-identity.

It therefore appears that the spot OU_X must be due to a new molecular species, possibly a third isomer. This conclusion cannot, however, be drawn from the paper-chromatograms without due precautions. When a supposedly pure substance produces one single spot, this is generally taken as an evidence of homogeneity, particularly when this behaviour can be replicated with different solvents or solvent mixtures. The converse is often tacitly assumed, that the production of multiple spots is an indication of inhomogeneity. During the last few years, however, it has become evident that a single pure substance may well under certain conditions give rise to more than one spot on the paper. When weak acids and bases are chromatographed with neutral solvents they will be partly ionized and the flow rate of the ionized and of the unionized form will very often differ [6-8,11]. Though this phenomenon usually gives rise to diffuse trails rather than to resolved spots, there are some reports to be found in the literature, in which the existence of multiple spots are attributed to ionization [11]. The fact that OU_{140} has been shown to be a weak acid [9] ($pK_a \approx 10.6$) merits a consideration of an explanation of the slow-moving spot OU_X along these lines. The question was settled by running some chromatograms with basic solvents. In Fig. 2 is shown a paper strip irrigated with ammonia/ethanol in which OU_{140} is present almost entirely as its anion. The observation that OU_X, recognizable by its very weak response to ferric chloride, has *not* increased in size at the expense of OU_{140} clearly indicates that the phenomenon cannot be interpreted as being due to ionization.

Another cause of "ghost spots" in paper-chromatography is particularly associated with water-rich, partially miscible solvent systems, such as for instance *iso*butanol>water. Due to either careless equilibration or to accidental drop in temperature during the irrigation the flowing solvent may contain traces of suspended water, which virtually constitutes a second phase, and the result may be the occurrence of multiple spots [10]. During the present work *iso*butanol and water were saturated one with the other by vigorous shaking, the phases were carefully separated by standing and these procedures as well as the development of the chromatograms were carried out in an air-thermostat at 25.0° ± 0.5. Water-logging of the paper should therefore be improbable. Finally the slow spot has also been found in chromatograms run with completely miscible solvents (*e. g.* Fig. 2), in which this phenomenon cannot arise.

Ghost spots are sometimes interpreted as being due to impurities in the paper (alkali salts [12], complex-forming heavy metals). Kennedy and Barker [13] have described a method of eliminating them in paper-chromatography of organic acids. The paper was washed with a 1 % aqueous solution of oxalic

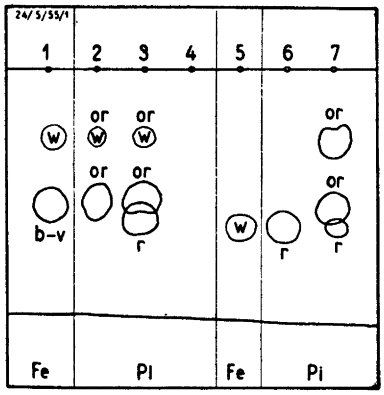


Fig. 2. Solvent ethanol-ammonia, 3½ h; spray-reagents 1 and 5 ferric chloride, 2—4 and 6—7 picryl chloride/ammonia; w weak, or orange, r red, b—v blue-violet. 1. OU_{140} 200 µg; 2. OU_{140} 100 µg; 3. OU_{140} 100 µg + OU_{72} 100 µg (mixture of isolated solids); 4. hydroxylammonium chloride 100 µg; 5. OU_{72} 200 µg; 6. OU_{72} 100 µg; 7. OU_{72}, 10 days old solution 1 %, 10 µl.

acid and subsequently rinsed with copious amounts of water and dried prior to the spotting-out. This treatment had, however, no effect on the double spot produced by OU_{140}, *vide* Fig. 3.

Finally "ghost spots" may arise when the solvent contains impurities, which can react with parts of the moving solute. Since one of the hydroxyureas (OU_{72}) readily forms carbamide-oximes with aldehydes [2], the *iso*butanol was carefully tested for a possible presence of aldehyde. The conventional test with 2,4-dinitrophenylhydrazine was negative.

With acidic flowing solvent (Table 3; Fig. 4) OU_{140} was found to give one spot only, detectable with picryl chloride/ammonia and by its blue-violet colour, when sprayed with ferric chloride. This spot seems to correspond to the principle spot in neutral and basic solvents. Although the absence of the slow-moving spot in acidic solvent may admittedly be correlated with the non-

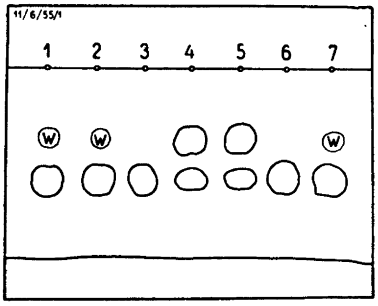


Fig. 3. Paper previously treated with oxalic acid. Solvent ethanol-ammonia, 2 hours; spray-reagent picryl chloride/ammonia; 100 µg substance in each spot. 1. and 2. OU_{140}; 3. OU^{II}_{140} (prepared according to process II); 4. and 5. Aged 1 % aqueous solution of OU_{72} (10 days) 10 µl; 6 as 3; 7 as 1.

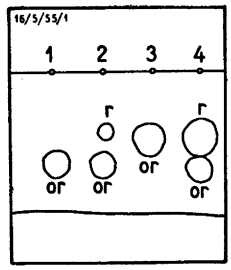


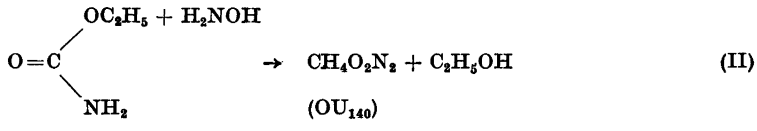
Fig. 4. Solvent ethanol-hydrochloric acid, 3 hours; spray-reagent picryl chloride/ammonia; r red, or orange. 1. OU_{140} 100 µg; 2. OU_{72} 100 µg; 3. hydroxylammonium chloride 100 µg; 4. OU_{72} 400 µg.

existence of the hydroxyurea anion at low pH values, it may as well be a result of insuffient resolution, *i. e.* the second spot is masked by the main spot.

It is seen that the order of the red OU_{72}-spot and the orange OU_{140}-spot on the chromatograms is reversed when the solvent is shifted from acidic to basic (*cf.* Tables 2 and 3 or Figs. 2 and 4). This is in accordance with the established acid-base-properties of the isomers [9], OU_{72} being a weak base, OU_{140} a weak acid. The ionic form is in either case the slower moving, in agreement with current rules [6].

A step further towards an interpretation of the slow-moving spot, OU_X, was made, when it was incidentally observed, that aqueous solutions of OU_{72}, when stored at room temperature and analyzed from time to time by paper-chromatography, at first produced the characteristic red spot (picrylchloride/ammonia), this then gradually vanished and two new spots appeared, which with respect to colour reaction and R_F-values were identical with the respective spots found for OU_{140}, Fig. 2, No. 7. It is seen that the slower moving spot OU_X is much more predominant in the aged solution of OU_{72} than in the isolated sample of OU_{140} (No. 2). It therefore appears a reasonable hypothesis that the spot OU_X is due to a new substance, possibly an isomer, which is present as a minor impurity in OU_{140} and is formed during the synthesis or during the isolation by heat-induced conversion of OU_{72}, *cf.* Fig. 6, Nos. 1—4.

In order to obtain further evidence for this general picture attention was drawn to a different route (II) for preparing the higher melting hydroxyurea OU_{140}. This has recently been reported by Runti and Deghenghi [14]. An alkaline solution of ethyl urethan and hydroxylamine was left standing for some days, then carefully neutralized and evaporated to dryness *in vacuo*. The hydroxyurea was extracted by means of absolute ethanol. In the hands of



(II)

the present author this product crystallized notably better than the product obtained by process (I) and showed a somewhat sharper melting point, suggesting a higher degree of purity. On paper-chromatograms developed with acidic (Fig. 5, Nos. 2—5) and basic solvent (Fig. 7, Nos. 2—5) one spot only was revealed. This was identical with the fast moving spot OU_{140} by R_F value and by colour reactions. Several fractions isolated from the reaction product of process (II) including the evaporated mother-liquors were analyzed without further purification, and in no one was any trace of OU_X detectable. These results corroborate the postulated explanation of the spot OU_X and further seem to indicate that in contrast to the ionic process (I) the molecular process (II) leads only to the formation of OU_{140}. Any simultaneous formation of OU_{72} would almost certainly have been revealed in the form of its conversion product OU_X. (The reaction mixture was exposed to 50° C for some hours during the evaporation.)

Additional information on the interrelationship of OU_{140}, OU_{72} and OU_X was obtained by the following experiments. A 1 % aqueous solution of freshly

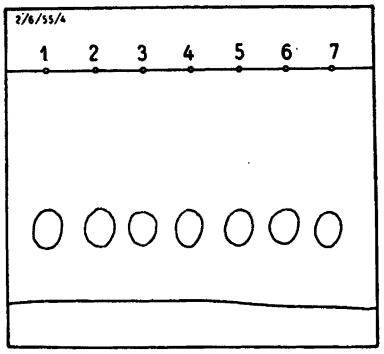


Fig. 5. Solvent ethanol-hydrochloric acid, 4 hours. Spray-reagent picryl chloride/ammonia; 100 µg in each spot. Colour orange. 1. OU_{140}; 2–5 OU^{II}_{140}, four successive fractions isolated from process II; 6. OU_{140}, 100 µg substance dissolved in 5 µl water, heated at 100° C for 10 minutes. 7. OU_{140}, 100 µg substance dissolved in 5 µl 1 N hydrochloric acid (20°), applied to paper.

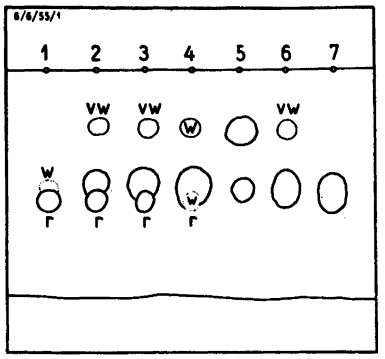


Fig. 6. Solvent ethanol-ammonia, 3 hours. Spray-reagent picryl chloride/ammonia; w weak, vw very weak; r red, other spots orange; 100 µg substance in each spot. 1. OU_{72} 1 % aqueous solution 10 µl; 2–4. the same solution heated at 100° C for 2, 5 and 10 minutes respectively; 5. OU_{72} 1 % aqueous solution aged at room temperature for 20 days; 6. OU_{140}; 7. OU^{II}_{140}.

isolated OU_{72} was divided into four parts. They were heated in a closed capillary in a steam bath for 0, 2, 5 and 10 minutes respectively. 10 µl of each batch was applied to the paper and chromatographed with a basic solvent mixture, Fig. 6, Nos 1—4. A similar experiment was made with extended heating periods, Fig. 9, Nos. 1—4. It is seen that heating has qualitatively the same effect as standing at room temperature. The characteristic red spot assigned to OU_{72} gradually disappears and OU_{140} and some OU_X are formed. The identification of the individual spots in Fig. 6 was accomplished by comparative runs of an aged solution of OU_{72} (room temperature, No. 5), of a pure sample of OU_{140} (No. 7), and of a sample of OU_{140}, containing some OU_X (No. 6). It appears that heating of the solution predominantly gives OU_{140}, whereas ageing at room temperature preferably gives OU_X (*cf.* Fig. 2, No. 7). In old

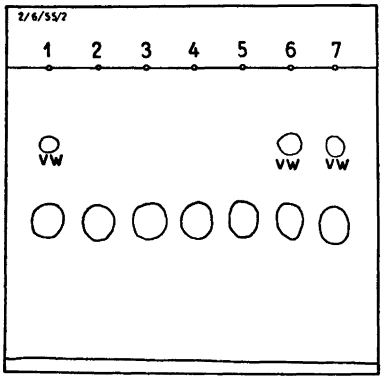


Fig. 7. Solvent ethanol-ammonia, 4 hours. Spray-reagent picryl chloride/ammonia; vw very weak. 1–7 as in Fig. 5.

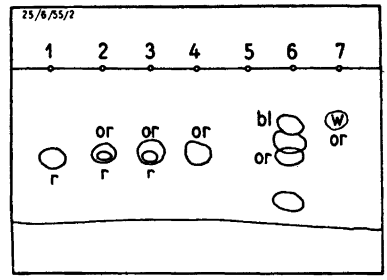
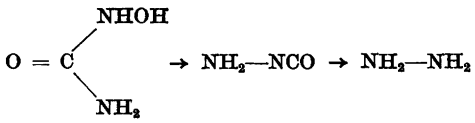


Fig. 8. Solvent ethanol-ammonia, 2 hours. Spray-reagent picryl chloride/ammonia. 1.—4. and 6. OU_{72} 1 % solution in 2 N ammonia, heated at 100° C for 0, 4, 6, 20 minutes and 20 hours respectively; 10 µl applied to paper in each case. 7. OU_{72} 1 % aqueous solution aged at room temperature for 40 days, 10 µl; r red, or orange, bl blue.

aqueous solutions of OU_{72} (40 days) only a weak OU_X spot is detectable, whereas OU_{72} and OU_{140} are completely destroyed (Fig. 8, No. 7).

At extreme pH values aqueous solutions of the hydroxyureas are very sensitive to heat. Thus in an acid solution of OU_{72} heated for one hour neither the starting material nor any conversion product was detectable (Fig. 9, Nos. 5—7). In an alkaline solution heated at 100° C for 20 minutes (Fig. 8, Nos. 1—4) all OU_{72} was converted to OU_{140} or decomposed. No OU_X was found. Further heating for 20 hours of the same solution gave rise to a rather complicated chromatogram (Fig. 8, No. 6) showing four substances, one of which was OU_{140}. The blue spot may be due to hydrazine, which is known [4] to produce a blue colour with picryl chloride/ammonia. Hydrazine may be formed by a Lossen-rearrangement of hydroxyurea followed by hydrolysis in the alkaline medium



This interesting possibility will be subjected to a closer examination.

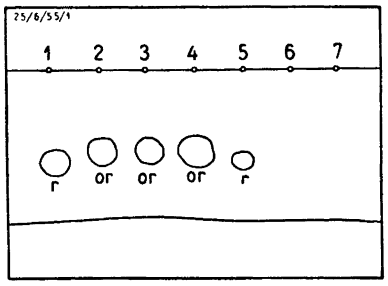


Fig. 9. Solvent ethanol-ammonia, 2 hours. Spray-reagent picryl chloride/ammonia. 1—4. OU_{72} 1 % aqueous solution heated at 100° C for 0, 30, 60 minutes and 20 hours respectively; 5. OU_{72} 1 % solution in 1 N hydrochloric acid heated at 100° C for 0 and 60 minutes and 20 hours respectively; 10 µl applied to paper.

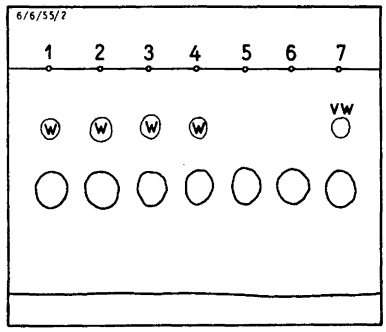


Fig. 10. Solvent ethanol-ammonia, 3 hours. Spray-reagent picryl chloride/ammonia. 1—4. OU_{140} 1 % aqueous solution heated at 100° C for 0, 2, 13 and 30 minutes, respectively; 5—7. OU^{II}_{140} 1 % aqueous solution heated at 100° C for 0, 10 and 30 minutes, respectively. 10 µl applied to paper.

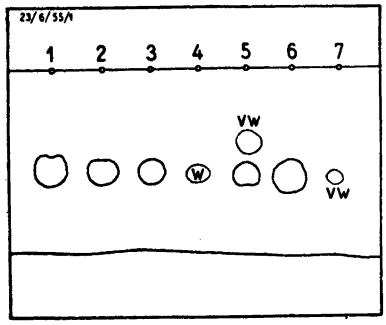


Fig. 11. Solvent ethanol-ammonia, 2½ hour. Spray-reagent picryl chloride/ammonia. 1—4. OU^{II}_{140} 1 % aqueous solution heated at 100° C for 0 and 30 minutes, 3 and 20 hours, respectively; 5—7. OU^{II}_{140} 1 % solution in 1 N hydrochloric acid, heated at 100° C for 30 minutes, 0 minutes and 3 hours, respectively; 10 µl applied.

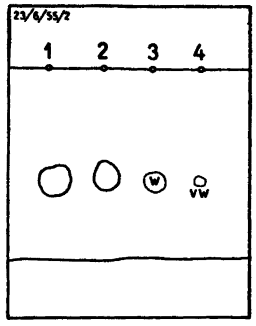
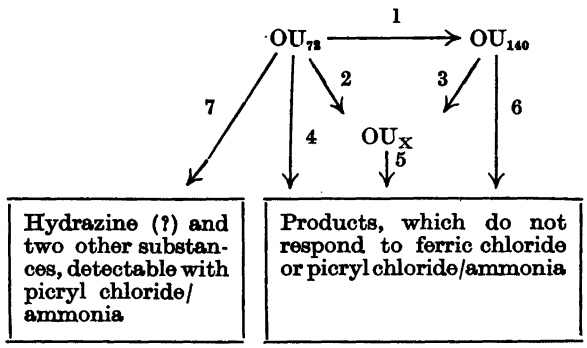


Fig. 12. Solvent ethanol-ammonia, 2½ hour. Spray-reagent picryl chloride/ammonia. 1—4. OU^{II}_{140}, 1 % solution in 2 N ammonia, heated at 100° C for 0 and 30 minutes, 3 and 20 hours respectively; 10 µl solution applied to paper.

The postulated[2] irreversibility of the conversion $OU_{72} \rightarrow OU_{140}$ was tested by a similar series of experiments in which OU_{140} was heated in neutral, acid and alkaline solution. A 1 % aqueous solution of pure OU_{140} was heated at 100°. After 20 hours heating the OU_{140}-spot was still discernible; neither OU_X nor any other conversion product was found (Fig. 11, Nos. 1—4). This experiment was repeated with a OU_{140}-sample containing OU_X (Fig. 10). The relative densities of the two spots are seen to remain unaffected by the heating of the solution and no novel spot is formed. A similar experiment with an acid solution of pure OU_{140} indicated that some OU_X was formed after 30 min. After a total of 3 h heating the OU_X spot had vanished and only traces of OU_{140} were still detectable (Fig. 11, Nos. 5—7). When an alkaline solution of pure OU_{140} was heated under similar conditions (Fig. 12, Nos. 1—4) the OU_{140} spot disappeared almost equally fast as in acid solution, but neither OU_X nor any other compound were found on the chromatogram.

The above results may be summarized in the following diagram



1 and 2 proceed simultaneously in neutral aqueous solution, 1 seems to predominate at 100° C; 2 at room temperature. In alkaline solution at 100° 1 proceeds rapidly, 2 and 3 do not seem to occur.

3 has been noticed only in acid solution heated to 100°.

4 takes place rapidly in acid solution at 100°, probably *via* 2—5, 1—3—5 or 1—6.

5 and 6 proceed slowly at 100° in neutral, rapidly in acid and alkaline solution.

7 occurs on prolonged heating of an alkaline aqueous solution.

There are no indications that any of the above processes can proceed in the opposite direction and especially no signs of tautomerism. It should, however, be remembered that paper-chromatography is a slow method of analysis. Rapid processes may therefore have escaped notice.

Ethanol-ammonia was preferred as the irrigation solvent-system, because it gives good resolution of the three spots with only 2—4 hours development. Addition of ammonia was indispensable, irrigation with ethanol-water of the same composition showed very poor separation of spots. In the alkaline solvent some OU_{72} will be converted during the development period, (*cf.* Ref. 15), this should be remembered when making quantitative estimates from the chromatograms. The neutral solvent system *iso*butanol-water showed no advantage in this respect, because a considerably longer development period was required (14—16 hours) in order to get good separations with this slower moving liquid, and more OU_{72} appeared to be converted on these conditions than during a short-time development with ethanol-ammonia. The application of ethanol-hydrochloric acid, in which OU_{72} is known to be perfectly stable [15] is limited to analyses of OU_{72}—OU_{140} mixtures, because OU_X is not separated from OU_{140} with this solvent mixture.

Attemps to isolate the substance responsible for the spot OU_X by preparative paper-partition chromatography are in progress.

MATERIALS, APPARATUS AND GENERAL TECHNIQUE

The isomeric hydroxyureas denoted OU_{72} and OU_{140}, respectively, without a roman numeral, were prepared according to the ionic process (I). Details have been published earlier [5]. The samples of the higher-melting isomer denoted OU^{II}_{140} were prepared strictly according to the directions given in the paper by Runti and Deghenghi [14] (process II). The melting interval of our sample was 135—140° (decomp.); (lit.[14] 139—141° C).

The samples were stored at 0—5° C under dry conditions. The lower melting isomer OU_{72} was used for the experiments within 2 weeks from the preparation of the sample. Unless otherwise stated freshly prepared solutions were employed.

*Solvents. Iso*butanol-water. Commercial, pure *iso*butanol was thoroughly shaken with an equal amount of water at 25° C and the phases were carefully separated. The chromatograms were equilibrated in an atmosphere, provided by the water-rich lower phase and developed with the upper phase, *iso*butanol > water.

Ethanol-ammonia was a mixture of 20 ml of *ca.* 10 N ammonia solution and 80 ml of 96 % ethanol.

Ethanol-hydrochloric acid was a mixture of 30 ml of 4 N hydrochloric acid and 70 ml of 96 % ethanol.

Spray Reagents. The ferric chloride solution was a 1 N aqueous solution of an analytical grade product.

Picryl chloride was prepared from picric acid and phosphorus pentachloride according to Jackson and Gazzolo [16]. It was employed in the form of a 1 % (w/v) solution in ethanol.

Picryl chloride/ammonia. The developed chromatogram was sprayed with the picryl chloride solution, mentioned above, allowed to dry in the air and subsequently exposed to the vapours of 10 N ammonia solution.

Paper Chromatographic Procedure. Whatman paper No. 1 was used throughout, without any previous treatment, unless otherwise stated. The sheets were normally 22 by 28 cm, except for chromatograms, developed with *iso*butanol-water, which required a length of 55 cm in the travelling direction. The chromatograms were run by the descending technique and the substances were applied to the paper, normally in the form of a 1 % solution, as circular spots along a marked starting line as usual. In most cases 10 μl solution containing 100 μg substance were used.

The development of the chromatograms was carried out in conventional all-glas chambers maintained at 25.0° ± 0.5 in an air-thermostat. With the *iso*butanol-water system as a developer the paper was equilibrated with the lower-phase atmosphere for 18 hours. With the ethanolic mixtures 2—3 hours sufficed, and the same mixture was used for equilibration and development.

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Kinetics and Equilibria in Flavoprotein Systems

III. The Effects of Chemical Modifications of the Apoprotein on the Dissociation and Reassociation of the Old Yellow Enzyme

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The dissociation of the old yellow enzyme (O.Y.E.) and its resynthesis from FMN and protein have been measured spectrophotometrically and fluorimetrically on the same solutions. The two methods were found to give equal results.

O.Y.E. and its apoenzyme have been chemically modified by functional group reagents and the effects on the binding of FMN have been studied. Specific acetylation of a small fraction of the 60 primary amino groups strongly decreased the rate of resynthesis of O.Y.E. and increased its rate of dissociation. However, the acetylated apoenzyme was able to bind one mole of FMN when excess FMN was added. The essentiality of amino groups for the tight binding of FMN has been confirmed by the use of other chemical reagents. Formaldehyde inhibition of the apoenzyme was reversible, except when very low concentrations of the apoenzyme were exposed to formaldehyde.

Specific substitution of a small fraction of the 24 tyrosyl groups with iodine strongly decreased the reassociation reaction and increased the dissociation reaction of O.Y.E. However, in the presence of excess FMN, an iodinated apoenzyme was able to bind one mole of FMN.

Iodinated and acetylated apoenzyme appeared homogeneous in the ultracentrifuge with practically the same sedimentation constants as for the unmodified protein.

FAD and a riboflavin diphosphate have been found to combine loosely with the apoprotein.

Atabrin, which has been shown to interfere with the activity of other yellow enzymes also appears to combine loosely with the apoenzyme of O.Y.E., as indicated by a quenching of the fluorescence of atabrin by the apoprotein.

Implications of some of these findings have been discussed.

The observed effects [1] of pH, anions, and temperature on the reaction

$$\text{FMN} + \text{apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{FMN} \cdot \text{apoprotein (Old Yellow Enzyme)}$$

suggested strongly the dominant role of electrostatic forces in the binding of

FMN to the protein. Furthermore, evidence was presented to show that the doubly anionic phosphate group of FMN in juxtaposition to positively charged amino groups may furnish an important part of the energy of attraction.

However, the kinetic studies did not give information about the attachment of the isoalloxazine nucleus to the protein [2]. In the present work we have attempted to identify combining sites of the protein by subjecting the enzyme and the apoprotein to functional group inhibitors and measuring the effects on the kinetics and equilibria of the FMN-apoprotein-O.Y.E. system.

Also included in this paper are some comparative experiments of the fluorimetric and spectrophotometric technique for measurements of association velocity constants.

A short communication of this study has already been presented [3].

MATERIALS AND METHODS

Pure preparations of O.Y.E. were necessary for quantitative experiments. Thus, considerable effort was spent in improving the isolation procedure. The partially crystalline preparations of Vestling [4] were still inhomogeneous in electrophoresis. By use of paper electrophoresis we obtained limited amounts of entirely homogeneous material, containing 1 mole of FMN per 72 000 g of protein. The absorption coefficient of O.Y.E. at 465 mμ was redetermined and found to be $\beta = 2.52 \times 10^7$ cm^2/mole, on the basis [5] of $\beta_{\text{FMN, 450 m}\mu} = 2.81 \times 10^7$. The details will be described in a forthcoming paper. For some qualitative experiments somewhat less pure material was used.

Flavin mononucleotide (FMN) was purified from a commercial preparation by paper electrophoresis, as described previously [6]. A solution of FMN kept in the dark at 0° was 50 % destroyed in about two weeks, but if kept in a frozen state, remained stable for months.

Flavin adenine dinucleotide (FAD) was kindly supplied by Drs. O. and E. Walaas in this institute; the sample was obtained from baker's yeast with paper ionophoresis used for the final purification.

Acetylation of the enzyme and apoenzyme was accomplished by the addition of 1 μl quantities of acetic anhydride to the protein solution buffered with 2.5 M sodium acetate and kept at 0°. Not more than 1—3 μl acetic anhydride were required for extensive acetylation of 5 ml of a 50 μM solution of the apoenzyme. The acetylation of amino groups was followed by Van Slyke aminonitrogen analyses [7], which were carried out at room temperature on dialyzed samples containing approximately 15 mg of protein. A fifteen minute reaction time was found sufficient to give maximum values.

The acetylation of phenolic groups was followed by means of Folin's phenol reagent [8], the protein being first denatured with lauryl sulfate as described by Miller [9].

Iodination of the enzyme and apoenzyme was carried out in 0.1 M phosphate pH 7.4 at 0° C. The iodinating agent was 0.1 M I_2 dissolved in alcohol, and 1 μl quantities were added to approximately 50 μM protein solutions. Tyrosine was estimated by the Millon-Lugg procedure [10], and the diiodotyrosine formed was calculated from the difference in tyrosine content before and after iodination. The formation of diiodotyrosine in the apoenzyme was also demonstrated qualitatively in hydrolysates by paper electrophoresis [11]. Monoiodotyrosine also reacts with the Millon reagent, in contrast to diiodotyrosine.

Azobenzene sulfonic acid reagent was prepared as described by Jorpes [12].

Fluorimetric experiments using the modified apoprotein were conducted to determine the following:

1. The association velocity constant, k_1, in the way previously described [6].

2. The total combining capacity of the modified apoprotein for FMN, and the equilibrium constant for the combination. In measurements of the combining capacity, increasing quantities of FMN were added to a given quantity

of apoprotein until no more FMN was coupled. Because most of the chemical modifications of the apoprotein resulted in an increased dissociation of the FMN-apoprotein, a considerable excess of FMN was sometimes necessary to saturate the protein. The experiments were less accurate when the equilibrium constant was high *. The equilibrium constant, K, was calculated from the amount of combination when the protein was only partially saturated with FMN.

3. The dissociation velocity constant. This could be determined directly when functional group reagents were added to the O.Y.E. However, when the apoprotein had been treated with such agents, k_2 was more readily obtained from the relation $k_2 = k_1 \times K$ **.

RESULTS

A comparison between spectrophotometric and fluorimetric measurements of the association velocity constant

Since our previous experiments [1,3,6] had been done with the fluorimetric technique it was of interest to examine whether the disappearance of the fluorescence when FMN combines with the apoprotein took place with the same rate as the shift of the absorption band from 445 to 465 mμ [13]. Since the greatest difference in the light absorption of FMN and O.Y.E. was found to be around 495 mμ this wavelength was chosen for the measurements. A highly sensitive recording spectrophotometer of our own construction was used, but the spectrophotometric measurements were nevertheless far less sensitive than the fluorimetric ones. Therefore, concentrations of FMN and apoenzyme which were considerably higher than applied in previous experiments [1,3] had to be used in order to determine the association velocity at the same concentrations of reactants with both methods. In the absence of inhibitors, these high concentrations around 3 μM gave too rapid reactions to be recorded with our apparatuses; thus, polyvalent anions were added to decrease the velocity. Easily measurable association rates were obtained using phosphate and versene buffers. The results are shown in Table 1. They show that the spectrophotometric and fluorimetric methods gave similar results, the variations being within the experimental errors. We may thus feel confident that the results obtained by the aid of fluorimetry in all probability are identical with those that would have been obtained by rapid spectrophotometry.

Functional group reagents

Practically all protein reagents react with thiol groups and their presence makes the test for the essentiality of other groups difficult. It was therefore a great advantage for the further investigation of this problem that the pure

* In our first experiments published in a preliminary communication [3], highly acetylated or iodinated apoproteins were studied. In these the dissociation constant was so high that the total combining capacity appeared to be decreased, because high enough concentrations of FMN to saturate the protein were not applied.

** For the meaning of k_1, k_2 and K in modified protein preparations see the discussion p. 1597.

Table 1. Spectrophotometric and fluorimetric measurements of the reaction apoenzyme + FMN $\xrightarrow{k_1}$ *O.Y.E. at pH 7.0, 23° C.*

Buffer		k_1, $M^{-1} \times sec^{-1}$	
		Spectrophotometric	Fluorimetric
Phosphate Versene	0.5 M 0.0016 M	2.5×10^4	2.0×10^4
Phosphate Versene	0.03 M 0.0016 M	10.0×10^4	10.9×10^4

apoenzyme as well as a preparation denatured by incubation with lauryl sulfate were found not to contain thiol groups. These preparations did not react with *p*-chloromercuribenzoate [14], as measured by the spectral change at 250 mμ, and gave a negative nitroprusside test. Further, typical thiol reagents did not affect the binding of FMN to the protein; Cu^{++}, Fe^{+++}, Hg^{++}, and Ag^{+} in concentrations as high as 10^{-3} M and incubation of the apoenzyme with 1.5×10^{-3} M *p*-chloromercuribenzoate for 72 hours at 0° C, pH 6.7, caused no inhibition of the "on" reaction.

The apoenzyme was also very resistant to strong oxidizing agents like periodate and hydrogen peroxide. Thus, 100 equivalents of periodate per mole of apoenzyme (protein concentration 15 μM) at pH 5.5 [11] caused no inhibition after two hours, and only after 72 hours was 50 % of the apoenzyme unable to couple with FMN. There was no effect of 0.1 M H_2O_2 after 30 minutes incubation at 25° C, pH 6.6.

Acetic anhydride is claimed to be the reagent of choice for the specific acetylation of amino groups on proteins [15]. Under the conditions used, the only other types of groups which could possibly react are sulfhydryl and phenolic hydroxyl. In the old yellow enzyme, which does not contain thiol groups, inhibition after acetylation could be due to blocking of essential amino groups or phenolic groups, or both. Fortunately, incubation of acetylated tyrosine at pH 11 for 5 minutes hydrolyzes this compound [15] without affecting acetylated amino groups. Therefore, the inhibition which remains after this alkaline hydrolysis of acetylated O.Y.E. can be attributed to acetylated amino groups alone.

Acetylation with acetic anhydride was found to inhibit the "on" reaction strongly, and none of this inhibition was reversed by incubation at pH 11. This alkalinity was in itself harmless to the protein. The Folin's phenol color was the same in the acetylated sample as in the control, within the experimental error (±5 %). It may therefore be concluded that acetic anhydride is specific for amino groups in the apoenzyme under the conditions used in our experiments. In order to determine quantitatively the importance of amino groups, the apoenzyme was subjected to different degrees of acetylation and k_1 determined for the different samples. It is shown in Fig. 1 that for example

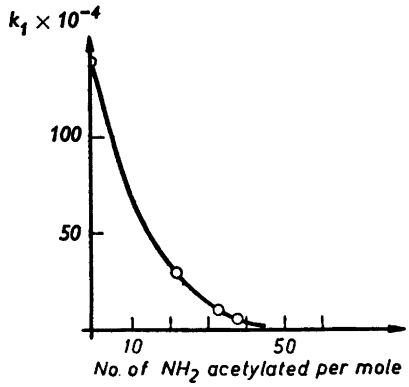


Fig. 1. The effect of acetylation of apoenzyme on k_1, as measured in 0.1 M glycine pH 9.0. 1 μl portions of acetic anhydride were added to 3 ml of a 27 μM solution of the apoenzyme in 2 M NaAc, pH 6.5, 0° C.

the acetylation of 22 of the 60 free amino groups resulted in 80 % decrease of the reaction velocity.

It was reported in the two previous papers in this series [1,6] that the dissociation constant, $K = k_2/k_1$, of the old yellow enzyme in water or non-dissociating electrolytes was less than 10^{-12} M between pH 4 and 10. This was mainly due to an extremely low "off" velocity constant (k_2) of the enzyme. Appreciable dissociation took place, however, in the presence of anions of strong acids, like chloride and sulfate. This effect was explained as a displacement of the phosphate group of FMN from its salt-like linkage to the amino groups of the protein. In view of these findings, it was interesting that an acetylated sample of the enzyme dissociated into FMN and protein even in the absence of chloride at pH 9.7, whereas no measurable dissociation in the absence, but an increased dissociation in the presence of chloride, took place at pH 5.3. A possible explanation is that, at pH 5.3, the number of positively charged, not acetylated amino groups was sufficient to keep the negatively charged FMN tightly bound, whereas this was not the case at pH 9.7, where the high pH would cause discharge of some amino groups.

The value of k_1 decreased continuously with increasing degree of acetylation (Fig. 1); k_2, on the other hand, had reached a maximum already when 7 of the 60 primary amino groups present in one molecule had been acetylated (See Table 2). Further, acetylation of up to 40 groups seemed not to increase but rather to decrease k_2 (however, see the discussion). The total combining capacity for FMN remained the same, 1 mole per mole, even in the most acetylated samples, where k_1 had decreased to 5 % of the value for the native apoprotein.

Formaldehyde is known to react with a number of groups in proteins and to form different crosslinkages between such groups. However, the reversible reaction that takes place with short exposure time and low concentrations of the reagent has been reported to involve mainly primary amino groups [15]. In

Table 2. The equilibrium constant (K) and dissociation velocity constant (k_2) as a function of the degree of acetylation.

No. of acetylated NH_2 groups per mole apoprotein	Per cent acetylated	$k_1 \times 10^{-6}$ sec^{-1} M^{-1}	$K \times 10^7$ M	$k_2 \times 10^2$ (from $k_2 = Kk_1$ sec^{-1})
0	0	1.4	$<10^{-5}$	0.00
7	12 (calc) *	0.79	0.23	1.8
21 **	35 »	0.30	0.63	1.9
21 **	35	0.29	0.52	1.5
26 ***	43 (calc)	0.19	0.74	1.4
26 ***	43 »	0.19	0.63	1.2
38	63	0.05	1.8	0.9
40	67 (calc)	0.04	2.7	1.1

* The degree of acetylation was calculated from Fig. 1 on the basis of k_1 obtained.
** Two different apoprotein preparations.
*** » » » »

the first studies with formaldehyde the old yellow enzyme or the apoenzyme were added at $t = 0$ to test solutions containing buffer, formaldehyde, and FMN (for the apoenzyme). The rate of dissociation or association was measured within the first few seconds. In this way, the "instantaneous" effect of formaldehyde on the dilute (0.1—1.0 μM) protein could be observed. Above pH 7 both the dissociation (k_1) and the association reaction (k_2) were affected by very low concentrations of formaldehyde. The effect on the dissociation was observed as an increased k_2 in the presence of sodium chloride. This is shown in Fig. 2. The effect of formaldehyde on the association reaction is shown in Fig. 3. It is seen that even 0.02 M formaldehyde had a large effect in the alkaline range, a much smaller one in the acidic range. This is what would be expected, since only uncharged amino groups combine with formaldehyde.

However, the reaction of the dilute apoenzyme with formaldehyde was not readily reversible. For instance, if the apoenzyme was allowed to react with formaldehyde in borate buffer at pH 9.3 for a few seconds, and the pH adjusted to 6.5 by the addition of hydrochloric acid, k_1 for this sample was much lower than for a control containing the same amount of formaldehyde, borate and sodium chloride, but not incubated at pH 9.3.

In order to obtain a reversible effect of formaldehyde, a more concentrated solution of apoenzyme (52 μM) was incubated at 0° C with 0.4 M formaldehyde in 0.1 M phosphate buffer, pH 8.0. The association reaction was tested in 0.1 M borate buffer, pH 9.0 and k_1 was found to decrease from 28×10^4 M^{-1} sec^{-1} to 6×10^4 M^{-1} sec^{-1} after 5 minutes and to 3×10^4 m^{-1} sec^{-1} after 20 minutes incubation. When the formaldehyde-treated apoenzyme was dialyzed against 0.03 M phosphate, pH 7.1, over night, k_1 was restored to 24×10^4 M^{-1} sec^{-1}. Thus, the reaction with formaldehyde around neutrality is reversible when a fairly high concentration of apoenzyme is used, but not when the concentration of apoenzyme is of the order of magnitude 0.1—1.0 μM. Also the sensitivity to formaldehyde inhibition was much less for concentrated samples

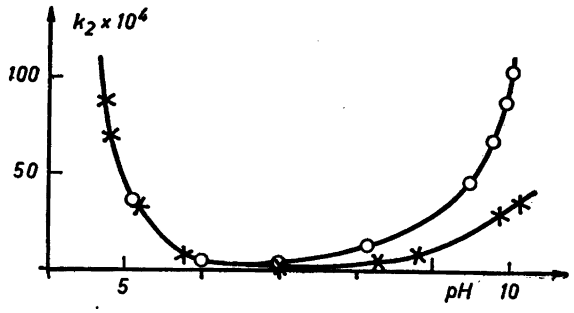


Fig. 2. The effect of 0.12 M formaldehyde on k_2 in 0.4 M sodium chloride, 23° C.

○○○○ 0.12 M formaldehyde
×××× no formaldehyde

50 μl O.Y.E., 64 μM, was added to 3 ml of a solution containing sodium chloride, 0.1 M sodium acetate + acetic acid (in the acidic range) or sodium hydroxide (in the alkaline range), and formaldehyde. k_2 was taken as the tangent at $t = 0$. pH was determined at the end of the experiment.

of the protein. For instance, a 52 μM solution of apoenzyme was not at all affected by 0.02 M formaldehyde even after 20 minutes incubation in borate buffer at pH 9, whereas the dilute samples of apoenzyme were affected strongly and instantaneously by 0.02 M formaldehyde (See Fig. 3).

Iodine, in all but one of the many proteins studied [15], reacts preferentially either in oxidation of thiol groups or substitution of tyrosine. The one exception is lysozyme where the imidazole groups were more readily iodinated than

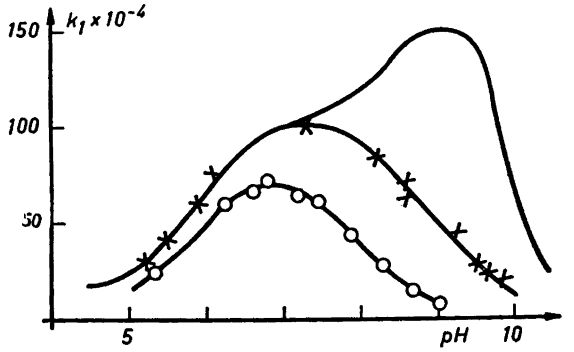


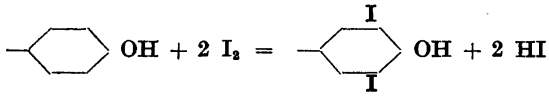
Fig. 3. The effect of formaldehyde on k_1 at 23° C.

– – – – without formaldehyde
×××× 0.02 M »
○○○○ 0.12 M »

15 μl apoenzyme, 42 × 10^{-6} M, was added to 3 ml of a solution of 0.15 μM FMN, 0.1 M sodium acetate, and formaldehyde. k_1 was measured within a few seconds. In the alkaline range the protein acted as buffer. pH was determined at the end of the experiment.

the tyrosyl groups [11]. Since the old yellow enzyme does not contain thiol groups, the iodine could be anticipated to enter into the 3- and 5-positions of tyrosine. Monoiodotyrosine has been proved to be a stable intermediate in the iodination of proteins, but in the cases investigated, relative small amounts of this compound were formed.

The iodination reaction proceeds according to the formula



Two moles of I_2 are thus required to form one molecule of diiodotyrosine.

An iodinated sample of O.Y.E. dissociated more rapidly in the presence of sodium chloride than did the control. The effect of iodination of the apoenzyme on k_1 and on k_2 (calculated from $k_2 = K\ k_1$) is shown in Fig. 4. Determination of the tyrosine content of the most iodinated sample, 13 moles of I_2 used per mole apoenzyme, showed that over 90 % of the iodine added had been used for the substitution reaction of tyrosine to form 6 moles of diiodotyrosine. The actual formation of diiodotyrosine was demonstrated qualitatively by paper chromatography of a hydrolysate [11]. The one mole of iodine per mole of enzyme which was not used for the formation of diiodotyrosine may have formed monoiodotyrosine or have caused some oxidation. In view of the great stability of the apoenzyme to other oxidizing agents, it is unlikely that a minor oxidation would cause inhibition. The iodine not present as diiodotyrosine would be sufficient only for the monosubstitution of one imidazole group; such a substitution appears unlikely. Even the most iodinated sample of the apoenzyme with 6 moles of diiodotyrosine per mole protein was able to quench the fluorescence of one mole of FMN per mole protein.

The sedimentation of acetylated and iodinated apoenzyme in the ultracentrifuge

An acetylated sample of the apoenzyme with $k_1 = 43 \times 10^4\ M^{-1}sec^{-1}$ as compared to $140 \times 10^4\ M^{-1}sec^{-1}$ for the control, appeared homogeneous in the ultracentrifuge with $S_{20} = 5.42$ as compared to $S_{20} = 5.36$ for the unmodified protein.

An iodinated sample containing about 8 moles of I_2 per mole protein with $k_1 = 19 \times 10^6$ likewise appeared homogeneous in the ultracentrifuge with $S_{20} = 5.61$.

The reaction of flavin adenine dinucleotide and riboflavin diphosphate with the apoenzyme

Warburg and Christian [16] have shown that the apoenzyme of O.Y.E. is nearly as enzymatically active with flavinadenine dinucleotide (FAD) as with FMN. It was therefore of interest to investigate whether FAD could form a nonfluorescent compound with the apoprotein. This was found to be the case, but the dissociation constant, K, of this compound around neutrality was

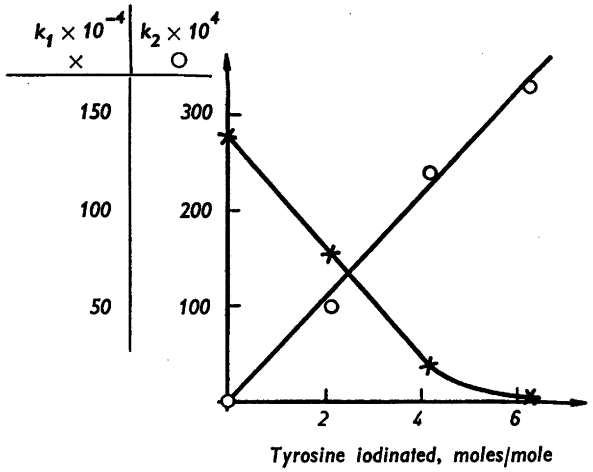


Fig. 4. The effect of iodination of apoenzyme on k_1 and k_2, as determined from rate of reassociation and equilibrium constant in 0.1 M glycine pH 9.0.

3 × 1.85 μl of I_2 in alcohol, 0.083 M, were added to 0.8 ml apoenzyme, 40.8 μM, which contained 23.8 moles tyrosine per mole apoenzyme. After iodination the tyrosine content was decreased to 17.5 moles tyrosine per mole apoenzyme. The apoenzyme was tested kinetically after each addition of 1.85 μl I_2.

some 200 000 times larger than for O.Y.E. The relatively high activity of FAD + apoprotein in Warburg's test system could have been due to the use of a large excess of FAD. With a large excess of riboflavin, the test system has been found to have a moderate activity [2]. The dissociation constant of riboflavin with the apoenzyme is ten times greater than for FAD + apoprotein. In Table 2, K, k_2, and k_1 for FMN, FAD, or riboflavin + apoprotein have been presented. It is seen that k_1, representing the number of successful collisions, is not grossly different for the three substances, but that the stability of the complexes formed, as expressed in k_2, is much greater for FMN-apoenzyme than for the two others. k_1 for FAD + apoenzyme has, like the FMN and riboflavin systems, a pH maximum around 9.

Some preliminary experiments with a riboflavin fraction from the paper electrophoresis of FMN, probably a riboflavin diphosphate, indicated that this compound formed a complex with the apoenzyme, with K, k_2, and k_1 of the same order of magnitude as for FAD and riboflavin.

Some preliminary observations

Phenylisocyanate, which primarily reacts with amino groups in proteins not containing thiol groups [15], was also found to decrease the "on" reaction. Incubation took place at pH 7.0, 0° C, with equal amounts (by weight) of reagent and protein. After 15 minutes with shaking, k_1 was decreased several fold. Unfortunately turbidity appeared gradually.

Diazonium compounds are known to react preferentially with imidazole and phenolic groups of proteins [15] and this reagent should therefore inhibit

Table 3. The reaction of the old yellow apoenzyme with FMN, FAD or riboflavin in 0.1 M glycine, pH 9.0, 23° C.

	K M	k_1 M^{-1} sec^{1-}	k_2 sec^{1-}
FMN	$<10^{-12}$	1.4×10^{6}	$<10^{-6}$
FAD	2×10^{-7}	0.2×10^{6}	0.04
Riboflavin	2×10^{-6}	0.1×10^{6}	0.2

the coupling of FMN to apoenzyme if tyrosyl groups were essential. When apoenzyme (28 μM) was allowed to react for 15 min at 0° C, pH 8.8, with azobenzene sulfonic acid (96 μM), k_1 for the treated sample was only 5 % of that of the control. In control experiments it was found that sulfanilic acid and nitrite added separately had no effect on k_1. The combining capacity was not determined in these experiments.

An acetylated sample of O.Y.E., when tested manometrically with TPN, Zwischenferment, glucose-6-phosphate, and oxygen at pH 7.4, was found to have lost an appreciable part of its activity. This was probably due to a partial dissociation of the acetylated enzyme under these conditions, as indicated by other experiments.

The enzymatic activity of O.Y.E. in the TPN-Zwischenferment system was decreased by iodination of the enzyme, probably again because of increased dissociation.

Walaas and Walaas [17] have demonstrated inhibition of the enzymatic activity of D-amino acid oxidase by structural analogues of the FAD molecule, and it was suggested that it could be due to inhibition of the coupling of FAD to the protein moiety. These investigators found that adenylic acid and ATP exerted 50 % inhibition in as low concentration as 5×10^{-4} M, whereas adenosine, adenine, and hypoxanthine were less inhibitory. In the case of the resynthesis of O.Y.E. from apoprotein and FMN we found that adenosine-3-phosphate, adenosine-5-phosphate, adenosine, adenine and hypoxanthine exerted about the same inhibition, a decrease of k_1 by 75 % in the concentration 4×10^{-3} M. Like the inhibition by polyvalent anions, the adenine inhibition was counteracted by chloride ions.

Atabrin, which has been shown to be a competitive inhibition of FMN in cytochrome reductase [18] and also to interfere with the activity of the FAD linked D-amino acid oxidase [19], was so strongly fluorescent that its possible inhibiting effect on the recombination reaction of FMN and apoenzyme could not be tested with our method. However, the fluorescence of atabrin was found to be somewhat quenched by the apoenzyme, and this quenching was a time reaction which could be recorded in our apparatus, in contrast to the instantaneous quenching caused by some ions. This shows that some reaction occurs between atabrin and the apoenzyme, which may resemble the binding of flavin coenzymes.

DISCUSSION

The partial modification of some groups in a protein, for example by acetylation or iodination, must necessarily lead to inhomogeneous preparations. Thus, if we acetylate 7 of 60 primary amino groups, 7 is an average value, and some molecules are more and some less acetylated. Furthermore, molecules with the same number of acetylated groups may be different. In our case, the FMN-binding groups may be acetylated in some molecules, in others not.

All this means that we cannot possibly expect the kinetics of the combination of FMN with such preparations to follow the second order for k_1 or the first order for k_2[1]. Because we determined the association velocity constant as the tangent of the curve at $t \approx 0$, our values of k_1 are maximum values, and the significance of changes in k_1 is all the more emphasized.

The experiments with the amino group modifying reagents support our earlier conclusion [1] that amino groups in juxtaposition to the phosphate group may serve as binding sites for FMN in the old yellow enzyme. Further, these groups appear to be more reactive than other amino groups. As can be seen from Fig. 1 the acetylation of half of the amino groups (30 of 60) decreases k_1 from 1.4×10^6 to (the maximum value) 0.14×10^6 which is 10 % of the original values. If the acetylation occurred at random, we would have expected 50 % decrease of k_1 if one $-NH_3^+$ and 75 % decrease if two $-NH_3^+$ groups were bound to FMN. This calculation is based on the assumption that the acetylation of non-binding $-NH_3^+$ groups does not affect k_1. This assumption may be valid, since the increased negative net charge of the molecule caused by acetylation must be largely neutralized by Na^+ ions from the solution of ionic strength 0.1. The total number of collisions between FMN- and acetylprotein molecules can therefore be expected to be roughly the same as for FMN + native apoprotein. The strong decrease of k_1, with small degree of acetylation, was not unexpected, since the combining site is likely to be located at the surface of the protein. Because riboflavin combines with the apoprotein, even a total acetylation of all amino groups should not abolish the combining capacity of 1 mole FMN per mole of apoprotein, unless the acetyl groups caused steric hindrance. It was actually observed that acetylation of 40 amino groups did not decrease the FMN combining capacity. This finding demonstrates the auxiliary character of the $PO_3^{-+}(-NH_3)_2$ bonds; they serve to stabilize the complex, but are not essential for the protein-isoalloxazine linkages, which quench the fluorescence, shift the absorption band from 445 to 465 mμ, and activate the coenzyme part of the complex.

As mentioned before, the dissociation constant, K, was calculated from one value of the degree of unsaturation of the modified protein in the presence of a small excess of FMN. Since a modified protein is non-homogeneous, the K determined in this way must be a very crude average of the K's for the different protein fractions and depending upon the concentrations of FMN and protein used. The same must apply for the value of k_2 that was calculated from $k_2 = k_1 K$, where k_1 is a maximum value. The data for K and k_2 given in Table 2 and for k_2 in Fig. 4 should therefore be regarded as very rough approximations. The sudden increase of k_2 after acetylation of 7 amino groups per

molecule must undoubtedly be correct (see Table 2), but the decrease in k_2 with increasing acetylation is probably insignificant (because of overestimated k_1).

The 3,5-iodination of tyrosyl groups caused a drastic decrease in k_1. Thus, iodination of 6 of the 24 tyrosine groups decreased k_1 from 1.4×10^6 to 0.06×10^6, and a measurable dissociation of the complex appeared. Since the phosphoric acid residue is bound by amino groups it seems very likely that tyrosine-OH groups serve as binding groups for the riboflavin part of FMN and that these tyrosine groups like the binding amino groups are preferentially exposed to chemical reagents. However, the iodination, as far as it could be followed experimentally, did not interfere with the combining capacity for FMN, but as in the case of acetylation, iodination increased the dissociation of the complex strongly. The 3,5-iodination leads to an increase of the acid dissociation constant of the tyrosine hydroxyl group [20], but not to a blocking of the hydroxyl group. It is therefore understandable that the iodination could lead to changes in k_1 and k_2, but not in the total FMN-combining capacity.

It is not yet possible to decide whether the tyrosine-riboflavin linkage is auxiliary, like the $-PO_3^{-+}(NH_3)_2$ bond, or whether it has essential character. There is thus still no direct experimental evidence to support Weber's [21] suggestion of tyrosine-OH quenching the fluorescence in the old yellow enzyme. But if this happens to be the case, the same linkage is likely to be responsible for the spectrophotometric band shift and the activation of the coenzyme.

The coupling between FMN and the apoprotein of the old yellow enzyme, which results in quenching of the fluorescence of FMN and displacement of its absorption band, appears to be a highly specific one in respect to both interacting molecules. From our data it is evident that such compounds as FAD, riboflavin, and our incompletely defined riboflavin diphosphate, which all contain the isoalloxazine nucleus, become more loosely bound to the apoprotein than FMN. This specific binding of one molecule of FMN to one molecule apoprotein is likely to result from complimentariness in structure of the combining region on the protein molecule [22]. This complimentariness may include both similarity in surface configuration and the juxtaposition of the special combining groups.

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Kinetics and Equilibria in Flavoprotein Systems

IV. The Standard Potential of the Old Yellow Enzyme of Yeast

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As part of a continuing study of the old yellow enzyme (OYE) of brewer's yeast by Theorell and co-workers, a re-determination of the standard potential of the highly purified enzyme has been made. The average value of E'_0 for OYE at pH 7 and 30° was found to be -0.123 ± 0.006 V. In the pH range, 4.8—8.9, the system showed a $\Delta E'_0 / \Delta$ pH slope of -0.06. Reductive titrations were made with reduced Safranine T as a reagent. The use of indigo disulfonate as a mediator led to acceptable stability of potentials. In all cases a flattening out of the titration curves was encountered after approximately 50 % reduction.

In addition, some slightly modified details of the isolation of crystalline OYE have been worked out.

In 1934 Theorell [1-3] reported the isolation of crystalline OYE by a procedure which involved the early steps of the methods of Warburg and Christian [4], followed by prolonged preparative electrophoresis. Characterization of this material [5] indicated one residue of riboflavin phosphate (FMN) per molecular weight of 75 000 g. It was possible to dissociate the FMN from the apoenzyme by acidification in the presence of ammonium sulfate and to reform the OYE by recombining the FMN with OY apoenzyme [3,5]. Since that time there have been surprisingly few studies of the metabolic importance of OYE. However, along with the recent upsurge of interest in metalloflavoproteins [6,7] a careful re-appraisal of OYE has been instituted [8-10].

EXPERIMENTAL

Isolation of crystalline OYE. The section to follow describes in detail the modified isolation procedure which led to a largely crystalline product which was approximately 85 % pure as shown by electrophoresis. This material was used in the potentiometric experiments. A complete analysis for metal ions and a re-evaluation of the extinction coefficients were not done. These matters will need to be cleared up in studies of pure OYE.

The procedure to be described involved the early steps of the Warburg and Christian procedure [4] and the alumina adsorption step of Weygand and Hocker [11]. Careful ammonium sulfate fractionation then led to a largely crystalline material.

Fresh bottom brewer's yeast from the Hamburger Bryggeriet, Stockholm, was washed several times by decantation with cold running tap water in a large sink. After washing, the yeast slurry was transferred to the cold room (5° C), chilled, and then centrifuged in an International Serum centrifuge for one hour at approximately 2 300 RPM. The thick gummy yeast was then air-dried after spreading on filter paper.

From 3 kg of fresh dried brewer's yeast, a Lebedev juice was prepared by adding 9 liters of distilled water at 37° C. Hand mixing was followed by slow mechanical stirring for 3 hours at 37° C. The container was then transferred to a refrigerated cooling bath at −15° and stirred rapidly until the temperature of the autolysate was 0 to −5°. The system was centrifuged for 1 ½ hours to yield 4 200 ml of a brown extract which was used immediately. The precipitation of the lead salts of proteins was accomplished by adding slowly with mechanical stirring at 0° 400 ml of lead subacetate solution (D.A.B. VI) per liter of Lebedev juice. Stirring was continued for one hour, and the preparation was usually stored overnight in the cold room. Centrifugation at 2 300 rpm separated lead proteinates. The excess lead was removed by adding at 0° with mechanical stirring 200 ml M/2 phosphate, pH 7, per liter of *original* Lebedev juice. After stirring for one hour the preparation was centrifuged to remove lead phosphates.

Acetone precipitations were carried out in the temperature range, 0 to −15° C. The supernatant liquid from above (volume, 5 300 ml) was cooled to 0°, and then one-half volume of cold acetone was added with mechanical stirring during one hour, and the temperature was allowed to fall to −15° as rapidly as possible. The rather copious light precipitate was allowed to settle for one hour, and then filtered by gravity at −15° to yield about 6 liters of a clear yellow filtrate. CO_2 was then bubbled in rapidly for one-half hour, following which 0.7 volume of acetone (referred to the lead phosphate supernate) were added slowly with mechanical stirring at −15°. After standing for one-half hour in the cold most of the yellow supernatant liquid could be decanted from the rather oily precipitated slurry of OYE. Centrifugation was followed by dissolving the crude OYE in about 500 ml cold distilled water. Considerable mixing of the oily, red-yellow material was required to effect solution. Centrifugation at low speed yielded a clear yellow solution.

Acetone precipitation was repeated *twice* more as follows: To the OYE solution at 0° 0.6 volume cold acetone was slowly added with careful mixing as the temperature was dropped rapidly to −15°. The OYE separates as a red-yellow oil, and the rather t urbid supernatant liquid can be decanted away. The OYE was dissolved in 200 to 400 ml cold distilled water and then centrifuged to remove light insoluble material.

These steps were followed by an adsorption on alumina C−γ [12]. To the OYE solution at 0° were added 1.5 volumes of alumina. After careful mixing and standing for about 30 minutes at 0°, the preparation was centrifuged. If the supernate was yellow in color, more alumina was added. About three or four additions of alumina were required. When all the OYE was adsorbed, the alumina was washed twice in the centrifuge at 0° with cold distilled water. Elution was accomplished at 0° with dilute NH_3 . $(NH_4)_2HPO_4$ (33 ml concentrated NH_4OH + 10 g $(NH_4)_2HPO_4$, diluted to 500 ml). Three or four elutions with about 2 volumes of eluant per volume of alumina were required. Most of the pink color remained on the alumina. The OYE solution was dialyzed in the cold room for 12 hours against several changes of distilled water.

Ammonium sulfate fractionation of the above solution was carried out in several ways, one of which went as follows: The pH was adjusted to 5.5 with 10 % acetic acid, and the system was brought to 0.45 saturation with solid ammonium sulfate, added at 0°. After standing for a short time a small amount of precipitate was centrifuged out. The system was then brought to 0.5 saturation and re-centrifuged. If any yellow material was precipitated, it was saved and re-used. Further fractionation in the range, 0.5 to 0.65 saturation was carried out by "dialyzing in" the ammonium sulfate. The calculated amounts of ammonium sulfate were slurried in water into a cellophane tube which was then placed in the OYE solution and gently rocked to equilibrium at 0°. In this way preparations which were largely crystalline were obtained. One such preparation was about 85 % pure as shown by electrophoresis in phosphate buffer at pH 7.8.

In two preparations carried out as described yields of 517 mg and 576 mg OYE were obtained. In another preparation a lower yield resulted (281 mg), presumably because of the quality of the dried yeast.

The recovery of OYE and its purity can be followed during isolation by determining the optical density at 280 mμ and 465 mμ. After one ammonium sulfate fractionation a given preparation showed an O.D. $_{280\ m\mu}$/O.D. $_{465\ m\mu}$ ratio of 18.8, while an earlier preparation at the same stage showed a ratio of 35.7. The best OYE preparation obtained showed a ratio of 12.7 after five recrystallizations. Dry weight analysis suggested a purity of 96.8 % based on the extinction coefficient value [5], $E_{465\ m\mu} = 1.098 \times 10^7$ cm²/mole. However, on electrophoresis this material was approximately 85 % pure. The implication is that a re-evaluation of the extinction coefficient is needed.

The OYE was stored in 0.7 saturated ammonium sulfate at 0°. About 12 hours before use, portions were dialyzed against water or buffer at 0° and then diluted to desired concentrations based on the value of $E_{465\ m\mu}$ given above and a molecular weight of 75 000.

POTENTIOMETRIC EXPERIMENTS

The only reported value for E'_0 of OYE is that of Kuhn and Boulanger [13]. In this report exact details of the purity of the OYE were not given, and the major interest was in the E'_0 values for a number of synthetic riboflavin analogs. Two reductive titrations of OYE with $Na_2S_2O_4$ were presented, and no indication of a sluggish behavior of OYE at smooth platinum electrodes was given. Hence, it seemed desirable to re-examine the potentiometric behavior of OYE, since a reliable E'_0 value is a requirement for calculations of the free energy change for interaction with other oxidationreduction systems.

In kinetic studies of the reaction:

$$\text{FMN} + \text{OY Apoenzyme} \rightleftharpoons \text{OYE}$$

Theorell and Nygaard [8-10] have evaluated the dissociation constant, K_0, for this reaction under a variety of conditions.

$$K_0 = \frac{[\text{FMN}]\ [\text{Protein}]}{[\text{OEY}]}$$

An approximate figure for K_0 of 10^{-8} indicates a rather tight binding of FMN to the apoenzyme. As can be seen from the following equation [14]:

$$E'_{0\ (\text{OYE})} = E'_{0\ (\text{FMN})} + \frac{RT}{nF} \ln \frac{K_0}{K_r}$$

an indication of the tightness of binding of $FMNH_2$ by apoenzyme could be arrived at if the standard potentials of OYE and FMN were well established. It would be extremely difficult experimentally to measure the dissociation of $FMNH_2$ from $OYEH_2$ if the binding is such that K_r would be about 10^{-12}.

In these studies it was also desired to investigate the effect of chloride ion on a partially reduced OYE system [10]. If chloride ion acts to dissociate FMN and/or $FMNH_2$, a striking effect on the potentiometric behavior should be noted.

For the potentiometric measurements a titration vessel similar to that used by Rodkey and Ball [17] was constructed. It is shown in Fig. 1, in which drawing only one smooth platinum electrode is included. The jacket temperature of the cell was maintained at 30 ± 0.05° by means of a circulating pump

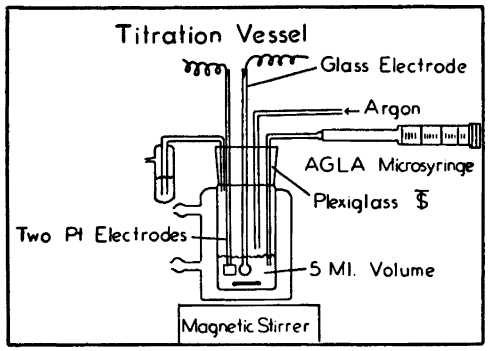


Fig. 1. Vessel for potentiometric titration of OYE.

and a water thermostat. A thermometer was placed in the line immediately adjacent to the water inlet of the cell jacket. Tank argon of high purity was passed through two bubble towers containing hypovanadous acid [16] to remove any traces of O_2 and then through a water tower. Rubber connections were avoided as much as possible and — where used — were painted with General Electric Glyptal Red Enamel, No. 1201. The bubble towers were glass-sealed to each other, and copper tubing was soldered to the argon tank valve and the joint painted with Glyptal. A painted rubber connection joined one end of the gas train to the copper tubing and the other end to the titration cell gas inlet. In use the cell was gassed at a rate of 2 to 3 bubbles per second for about one hour before titration was begun. The contents were stirred continuously with a magnetic stirrer. The inlet tubes were placed in carefully bored holes in a Plexiglas stopper ground to fit a 24/40 standard taper joint. The holes were countersunk to a depth of about 5 mm from the top so that thin gum rubber tubing sleeves could be placed on the glass tubes. Effective gas seals were made in these wells with a drop of water or glycerol.

The glass electrode was a *new* one (Catalog No. 120 B) ordered from Radiometer, Copenhagen. It had a 3 mm stem and bulb diameter and was the seventh electrode tested before satisfactorily reproducible potentials were obtained. It is the opinion of the author that commercial glass electrodes more than a year or two old cannot be expected to perform properly. The use of the glass electrode eliminated liquid junction potentials from consideration but necessitated the use of an electronic potentiometer. The instrument used was the Electronic Potentiometer, Type PHM 3a made by Radiometer. It was found desirable not to use the pH scale and temperature compensator circuit, but rather to make all measurements at 30° with the millivolt scale of the instrument. In this way potential readings on standard buffers made with the glass electrode *versus* a saturated calomel electrode agreed within 0.001 V from day to day.

To check the performance of the glass electrode and to make possible accurate pH readings of titration systems, three standard reference solutions were prepared with the greatest care [17]: (1) 0.100 M HCl, pH 1.084, 30°; (2) 0.05 M

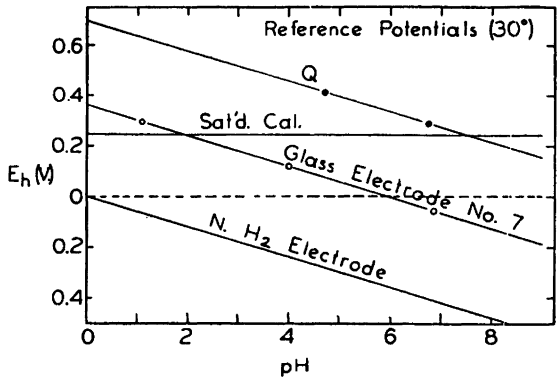


Fig. 2. Reference potentials. Solid lines are drawn with theoretical slopes of −0.06 V/pH unit. Open circles represent potentials measured with the glass electrode vs. *a saturated calomel electrode. Solid circles: glass electrode* vs. *quinhydrone electrode.*

potassium acid phthalate, pH 4.015, 30°; 0.025 M KH_2PO_4 — 0.025 M $Na_2HPO_4 . 2H_2O$, pH 6.848, 30°. When the glass electrode was checked against the saturated calomel electrode at these pH's and against the quinhydrone electrode at pH 4.70 and 6.85, its behavior was essentially perfect, in accordance with the expected $\Delta E_h/\Delta$pH value of −0.06 V per pH unit at 30°. It is estimated that recorded pH values measured at 30° were accurate to ± 0.03 pH unit. The potential scale of the potentiometer allowed readings accurate to ± 0.0001 V.

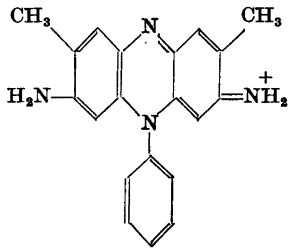
The microburette was the Agla Micrometer Syringe, Burroughs-Welcome and Co., London, which had a capacity of 0.5 ml and could be read to ±0.0005 ml. The microburette was fitted with a tip about 10 cm long, finely drawn out at the end. The drawing indicates that the end of the tip was placed below the surface of the liquid in the cell. The tip of the microburette was inserted through a moist gum rubber sleeve which formed a gas-tight seal under the 2 cm hydrostatic head maintained in the gas exist water trap.

The gas tight characteristics of the system were demonstrated on numerous occasions when partially reduced $OYEH_2$ systems were maintained for 3 to 4 hours at essentially constant potential. In pilot experiments leucomethylene blue was also successfully held at constant potential for several hours. On the admission of air to such systems the potential quickly shot up about 300 mV to values near the initial ones.

In Fig. 2 are presented the calibration data for the glass electrode, which carries the number 7 to indicate that its six predecessors had failed. The solid lines are theoretical lines drawn with a −0.06 slope, and the circles are experimental. The value for the saturated calomel electrode at 30° was taken to be +0.242 V. It is obvious that at pH 6 no corrections of the potentials shown by the glass electrode were required to convert them to E_h values.

REDUCTIVE TITRATIONS OF OYE WITH REDUCED SAFRANINE T

In preparing to carry out reductive titrations some thought was devoted to appropriate negative dyes as reducing agents. Through the kindness of Professor W. Mansfield Clark, The Johns Hopkins University, Baltimore, several valuable dyes were made available for this study. They included Safranine T, Safranine B, Induline Scarlet, Rosindone Sulfonic Acid ≠6, Rosinduline GG, phthiocol and several others. From this group phthiocol (2-hydroxy-3-methyl-1,4-naphthoquinone; E'_0, pH 7 and 30° = −0.180 V) and Safranine T (a phenazonium dye as shown in the formula; E'_0, pH 7 and 30° = −0.289 V) were slected [17].



Safranine T

Several pilot titrations were done with reduced phthiocol, following which the results to be presented were obtained with reduced Safranine T. The dyes were reduced immediately before use in a small two-vessel apparatus so arranged that tank H_2 could be bubbled through the two vessels to out-gas the system thoroughly, following which a bit of platinized asbestos was quickly admitted to the first vessel. As soon as reduction was complete (several minutes), partial closing of a by-pass valve between the two reducing vessels forced the nearby colorless reduced dye through a sintered glass filter stick into the second vessel, where it was kept under H_2 flow. The tip of the Agla microburette could then be inserted and the syringe rinsed several times with reduced dye solution before being filled for use in titration. Reduced Safranine T solutions seemed to be nearly stable for about an hour, after which they began to develop a very noticeable yellow fluorescence which undoubtedly changed their reducing titer.

In early experiments to test the apparatus reductive titrations of $K_3Fe(CN)_6$ and methylene blue were performed with reduced phthiocol as reductant It appeared necessary to "titrate out" residual traces of dissolved O_2 by adding small amounts of reduced dye and waiting for several minutes while the potentials fell rapidly about 200 mV or so. In the titrations of OYE, solutions of a final concentration of about 5×10^{-5} M were used. On the basis of a final volume of 7.0 ml and a molecular weight for OYE of 75 000, this amounted to about 20 mg per titration. Two things soon became apparent: 1) The OYE was electromotively sluggish at a smooth Pt electrode; and 2) reduced phthiocol appeared not to be "negative" enough to reduce the system completely. A few attempts to use de-aerated solutions of $Na_2S_2O_4$ as a reducing agent led to

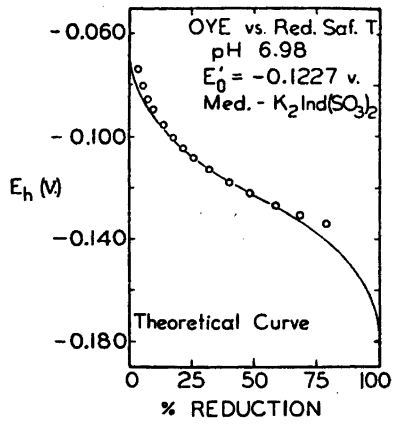


Fig. 3. Titration of OYE with reduced Safranine T. Potassium indigodisulfonate as mediator. OYE = 3 × 10^{-5} M. Mediator = 1.5 × 10^{-6} M. 0.05 M Phosphate buffer, pH 6.98. Argon atmosphere. 30°. Circles are experimental points. Curve is theoretical for two-electron change.

nothing simulating either stable potentials or theoretical behavior. It is true that reduction could be accomplished (accompanied by bleaching of the yellow color), but a satisfactory titration curve could not be obtained.

Accordingly, the use of potassium indigo disulfonate (E'_0 at pH 7, 30° = −0.125 V) as a mediator at 1/20 of the molar concentration of OYE was tried, and reduced Safranine T was used as reductant. In Fig. 3 is shown an experiment which is quite typical of those titrations in which acceptably stable potentials were achieved (drifts less than 0.0001 V per minute). In every case the early potentials after gassing for about one hour were about 200 mV more positive than those reached rather quickly after the first small additions of reduced Safranine T. It was possible to detect the end of the "titrating out" of residual O_2 by noting the arrival of stable potentials and agreement between the two Pt electrodes.

The curve in Fig. 3 is a theoretical curve for a 2-electron change. It can be seen that the points were reasonably well behaved until about 50 % reduction was reached, at which point a very considerable flattening out was repeatedly encountered, even though the reduced Safranine T should be plenty "negative" to allow the completion of the titration. It soon became apparent that the E'_0 values were going to be lower by about 0.07 V. than those previously reported by Kuhn and Boulanger [13] and that a distinctly nontheoretical behavior was being manifest. Analysis of the titration curves by the method of Reed and Berkson [18] as modified by Clark and Perkins [19], which involves the rearrangement of the equation for the potential into a linear slope — intercept form, revealed a profound deviation from ideal behavior.

A summary of data from eight acceptable titrations is presented in Table 1. The rather large deviations are indicative of the difficulty of establishing equilibrium between oxidized and reduced OYE at an inert electrode. The

Table 1. Old Yellow Enzyme. Calculation of E'_0 values at pH 7, 30°.

pH	E'_0 (V)	E'_0 at pH 7 (V)
4.80	+0.0168	−0.1152
6.00	−0.0747	−0.1347
6.22	−0.0856	−0.1324
6.75	−0.1033	−0.1183
6.98	−0.1227	−0.1239
7.00	−0.1206	−0.1206
8.15	−0.1919	−0.1229
8.90	−0.2335	−0.1195
	Average	−0.1234±0.0064 V.

average value, − 0.123 V is 0.064 V lower than that previously reported. The effect is to bring the OYE system somewhat closer to the pyridine nucleotides.

In Table 2 are presented several E'_0 values for tentative reference. It can be seen that the effect of binding FMN to its apoenzyme is quite comparable to that of binding DPN^+ to yeast alcohol dehydrogenase, as calculated by Theorell and Bonnichsen [20].

Several reductive titrations were also carried out with riboflavin and FMN. Here, substantial agreement with Kuhn and Boulanger was noted. In Fig. 4 the E'_0 values obtained for riboflavin and FMN are shown, and the E'_0—pH relationship for OYE is given. The open circles represent experiments in which potassium indigo disulfonate was used as a mediator. The solid circle is a single experiment in which no mediator was used. These results indicate clearly that no proton dissociation is involved in the oxidation-reduction of OYE in the pH range, 4.7 to 8.9. The "flattening out" of the titration curves was also encountered in titrations of riboflavin and FMN, but stable potentials were rapidly attained indicating that the "sluggishness" exhibited by OYE is probably due to steric influences of the large protein moiety.

It is not possible at this time to asses properly the significance of the failure of OYE, FMN and riboflavin to be completely reduced. It can be suggested that the "too positive" potentials were indicative of an essential instability of the reductants, making the term, 0.03 log[oxidant] / [reductant], larger numerically than it should be. Clark and Perkins [19] and Stiehler *et al.*[21] noted an

Table 2. E'_0 values at pH 7, 30°.

O_2	+0.81 V	
Cytochrome *c*	+0.262 V	
Cytochrome *b*	0	
OYE	−0.123 V	0.063 V
FMN	−0.186 V	
ADH—DPN^+	−0.240 V	0.078 V
DPN^+	−0.318 V	
H_2	−0.420 V	

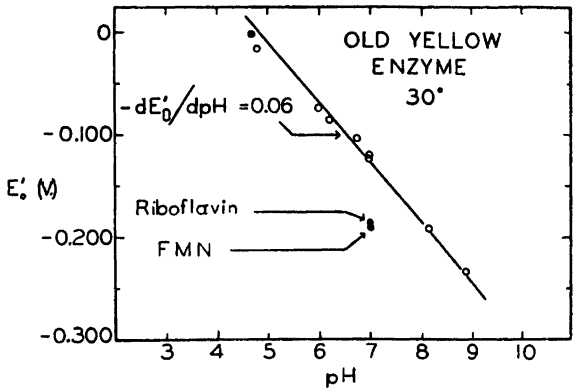


Fig. 4. E'_0-pH Curve for OYE. 30°. Acetate, phosphate, glycine or Veronal buffers: 0.05 M. Open circles with 1/20 molar equivalent of potassium indigodisulfonate. Solid circle without mediator. E'_0 for riboflavin (−0.185 V, pH 7) and for FMN (−0.190 V, pH 7).

apparent similar instability of the reduced forms of the phenazine dyes, neutral red and several of the safranines. It may be that the cell maintains its flavin materials in the oxidized state to avoid such inherent instability.

EFFECT OF CHLORIDE

Recently Theorell *et al.*[10] have suggested an interesting possible effect of chloride ion with respect to the dissociation of certain coenzymes from their apoenzymes.

It appeared possible in these potentiometric studies to reduce an OYE system partially and then add O_2-free saturated KCl with the microburette. When this was done at pH 4.77, an immediate effect was noted. The potentials became erratic, and disagreement between the Pt electrodes developed. Presently a turbidity was noticed accompanied by a rapid negative drift of about 0.06 V. After an hour the system contained a considerable quantity of precipitated protein, and the solution was a different shade of yellow-red, suggesting that FMN had been dissociated from its apoenzyme by the action of chloride ion. The final Cl^- concentration in this experiment was 0.39 M. At pH 9.35 a similar effect of Cl^- was observed. It seems clear that these experiments demonstrated a dissociating effect of Cl^- on OYE.

If E'_0 values for OYE and FMN of −0.123 V and −0.190 V are used and a value of 10^{-8} M is taken for K_0, one arrives at a value for K_r of 6×10^{-11} M. This approximate value of course indicates an extremely tight binding of $FMNH_2$ to its apoenzyme, a situation which is probably duplicated by $FADH_2$-apoenzyme complexes.

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Viscosimetric Determination of the "Michaelis Constant" for Proteolytic Enzymes and its Use for the Determination of Reaction Mechanisms

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A method is given for the viscosimetrical determination of the "Michaelis constant" for proteolytic enzymes with gelatine as substrate. The procedure is demonstrated with trypsin but primarily intended for very weak enzyme preparations. Using gelatine with a high intrinsic viscosity — which can easily be obtained by fractionating precipitation of solutions of commercial gelatine with ethyl alcohol — it is possible to determine "Michaelis constants" with values as low as about 0.1 per cent. The use of "Michaelis constants" at various pH values for finding out the mechanism by which an enzyme and its substrate combine, is discussed.

The viscosimetric method is more sensitive than other methods for the determination of the activity of proteolytic enzymes. Unfortunately, gelatine is almost the only substance that can be used as substrate, since a high viscosity is needed. This is a draw-back in investigations of reaction mechanisms, as substrates with only one type of linkages would be preferable, but such high polymeric, water soluble substances are not easily available [1].

The viscosimetric method is of particular interest for investigations of those proteolytic enzymes which are so weak that other methods cannot be applied. Several such enzymes with great physiological interest are known, *e. g.*, the sequence of proteolytic enzymes appearing and disappearing in the early development of a fertilized sea urchin egg, recently studied by one of us [2,3], and the proteolytic enzymes of many bacteria.

The investigations hitherto carried out on the weak proteolytic enzymes have almost exclusively concerned their occurrence and activity-pH-curve. However, it would also be of great interest to study their reaction mechanisms. Two different problems then arise: the specificity of the enzymes as to the amino acids whose linkages can be split, and the protolytic form (acid or base form) of the enzymes (and possibly also of the substrate) which can enter into the enzyme-substrate compound. The first problem will not be considered here, as gelatine was the only available substrate.

THEORETICAL AND EXPERIMENTAL

The method for finding out the mechanism by which an enzyme combines with its substrate was originally worked out for saccharase. The first interpretation of a chemical reaction with the enzyme molecule was given by Henri [4] as early as 1902: the enzyme and the substrate combine according to the law of mass action; the enzyme and an inhibitor combine also according to the law of mass action and the enzyme-substrate compound is the instable compound which is split *. Hudson [5], and Michaelis and Davidsohn [6] introduced the theory that the enzymes are amphoteric electrolytes, and that the branches of the activity-pH-curve correspond to two dissociation curves for the enzyme; shortly thereafter Michaelis and Menten [7] took up ** Henri's theory about the enzyme-substrate compound and its dissociation constant. This constant is nowadays understood as a combination of rate constants [8]. The two theories, one about the influence of pH and the other about the influence of the substrate concentration, were combined by Myrbäck [9,15], who gave the following equation in 1926

$$\frac{v_{max}}{v} = \frac{1}{(1 + K_a/[H^+] \cdot [1 + K_m/[S] \cdot (1 + K_b/[OH^-])]} \qquad (1)$$

* Henri [4]: "Je suppose qu'une partie z de ce ferment se combine avec une partie du corps à dédoubler; qu'une autre partie y du ferment se combine avec une partie des produits de l'hydrolyse; et enfin qu'il reste une portion X du ferment qui reste libre. Je suppose, en plus, que ces combinations se produisent suivant la loi de l'action des masses. On obtient ainsi les trois équations suivantes:

$$(a-x)X = \frac{1}{m} z \qquad Xx = \frac{1}{n} y \qquad \Phi = X + y + z$$

On peut supposer .... que la combinaison z entre les corps à dédoubler et le ferment est une combinaison intermediaire instable, qui se décompose en régénérant une partie du ferment. Dans ce cas la vitesse de la reaction sera proportionelle à la quantité de cette combinaison z; donc on aura

$$\frac{dx}{dt} = \frac{K\Phi\ (a-x)}{1 + m\ (a-x) + nx}."$$

This is the correct equation for the competitive inhibition. If the denotations now in use are substituted for Henri's notations: $a-x = [S]$, $x = [I]$, $\Phi = [E_{total}]$, $X = [E]$, $y = [EI]$, $z = [ES]$, $1/m = K_m$, $1/n = K_I$ and $K\Phi = V/K_m$, his equations are written

$$[S] \cdot [E] = K_m\ [ES], \quad [I] \cdot [E] = K_I\ [EI]', \quad [E_{total}] = [E] + [EI] + [ES]$$

$$v_i = \frac{V\ [S]/K_m}{1 + [S]/K_m + [I]/K_I}$$

For graphical computations the last equation is nowadays preferably written in the following form [7]

$$\frac{1}{v_i} = \frac{1}{V} + \frac{K_m}{V} \cdot \left((1 + \frac{[I]}{K_I}\right) \cdot \frac{1}{[S]}$$

** Michaelis and Menten [7]: "Die untersuchungen von Henri sind deshalb bedeutungsvoll, weil es ihm gelang, von rationellen Vorstellungen über das Wesen der Fermentwirkung ausgehend zu einer mathematischen Formulierung des Ganges der Fermentwirkung zu gelangen, die sich den Tatsachen in vielen Punkten ganz gut anschloss. Von diesen Vorstellungen Henris werden wir auch in dieser Arbeit ausgehen."

Myrbäck also drew attention to the following facts. The enzymes are ampholytes; they can take up and give off hydrogen ions, and this exchange of protons (protolysis) can — in the case of saccharase — be written in the following way [13] (N stands for a basic group containing nitrogen).

$$\left.\begin{matrix} \text{⁻EN:} \\ \text{HEN:} \end{matrix}\right\} + 2\text{H}^+ \rightleftharpoons \left.\begin{matrix} \text{⁻ENH}^+ \\ \text{HEN:} \end{matrix}\right\} + \text{H}^+ \rightleftharpoons \text{HENH}^+ \qquad (2)$$

Only those protolytic forms of this enzyme which have a lone electron pair at the nitrogen can combine with the substrate: hydrogen ions will thus cause a competitive inhibition of the enzyme in sufficiently acid solutions. One consequence of this competitive inhibition in the case of saccharase is that the acid branch of the pH curve depends on the substrate concentration whereas the apparent pH value for optimum activity also depends on the substrate concentration.

In some enzyme systems, the substrate may be an acid or a base or even an ampholyte. It is not unusual then that only one of the protolytic forms of the substrate can be acted upon by the enzyme.

It is evident that the "Michaelis constant", *i. e.*, the substrate concentration at which half of the enzyme is bound to the substrate, is influenced by pH, if only a restricted number of protolytic forms of an enzyme and its substrate can combine. It is therefore possible to find out the mechanism by which the enzyme combines with the substrate, *i. e.*, the protolytic (acid or base) forms which can combine, by measuring the enzymatic activity at various pH values and various substrate concentrations [9–16]. In a few cases the enzyme combines not with one but with, *e. g.*, two molecules of the substrate, and this reaction mechanism can also be found out by such measurements [17].

It has been emphasized by Myrbäck that it is very important to distinguish between the apparent and the true "Michaelis constant". The apparent value of "Michaelis constant" is the value obtained in the usual way without consideration of the protolytic (acid or base) form of the enzyme and substrate; it is often valid only for the particular pH at which it was measured; the true value of the "Michaelis constant" is calculated with regard to the protolytic forms which can actually combine and is hence independent of pH. It can be defined [14] by *e. g.*, the following equation:

$$K_m = \frac{[\text{S}]\,[:\text{NEH}]}{[\text{S:NEH}]} \qquad (3)$$

It is evident that the rather common habit of determining the "Michaelis constant" at just the pH optimum for activity is of hardly any interest other than that it gives us some information about how high the substrate concentration shall be in an ordinary determination of enzymic activity if almost the whole quantity of enzyme shall be utilized and if small variations in the substrate concentrations shall be insignificant.

The viscosimetric method seems not to have been used previously for the determination of the "Michaelis constant" for any enzyme and this investiga-

tion was made in order to find out the conditions for determining the apparent value of "Michaelis constant" for proteolytic enzymes, using a viscosimetric method. If such a method can be applied, it will be possible to investigate the mechanism for proteolytic enzymes which are too weak to be assayed by other methods.

A prerequisite for the application of a viscosimetric method is that the influence of the substrate concentration on the result of the viscosimetrical measurements can be eliminated. This is possible according to the theory for the viscosimetrical determination of enzymic activity, published by one of us [18].

One general difficulty in the viscosimetrical determination of a "Michaelis constant" is that it is necessary to get a substrate with a high intrinsic viscosity [19-21] which implies a high degree of polymerization, as the solutions actually used must have a sufficiently high viscosity to give results of acceptable accuracy, even if the concentration of the substrate is lowered so much that it approaches the value of the "Michaelis constant". This difficulty is noticeable in the case of proteolytic enzymes.

Enzyme

It would have been tempting to start immediately with, *e. g.*, one of the proteolytic enzymes in newly fertilized eggs, which have been detected by one of us [2,3]. However, the way in which these enzymes appear and disappear makes it plausible to assume the presence of some inhibitor, and it is evident that the crude preparations previously used must be purified before the properties of these enzymes are studied further. As already pointed out, the aim of this investigation is more general: to find the conditions for determining the "Michaelis constant" for proteolytic enzymes viscosimetrically. We therefore considered it appropriate to use a pure, crystalline enzyme, trypsin, for this first investigation. As substrate we used a high polymer gelatine preparation.

High polymer gelatine

It has already been mentioned that it is important to get a gelatine with a high intrinsic viscosity, and there are two reasons for this. The first is the following. If two gelatine preparations of different intrinsic viscosity are used for making up solutions of the same viscosity, the solution containing the gelatine with the higher intrinsic viscosity is more dilute than the other solution. If, in determining the proteolytic activity of enzyme solutions, we shall have the same drop in viscosity in unit time with both solutions, we must have a more dilute enzyme solution when we use the substrate solution with the more high molecular gelatine and the lower concentration. This can be concluded from the formula derived by one of us [18]

$$A = c_s^2 \frac{\mathrm{d}\ 1/\eta_{sp}}{\mathrm{d}t} \tag{4}$$

where A is the enzyme activity, c_s is the substrate concentration in the reaction mixture, and η_{sp} is the specific viscosity of the reaction mixture at the time t.

Hence, by choosing a gelatine preparation with a higher intrinsic viscosity than usual we can work with more dilute substrate solutions and thereby increase the sensitivity of the method so that we can either measure the activity of enzyme solutions which otherwise are too weak to be assayed or we can carry out the measurement in much shorter time than otherwise or we can get a higher accuracy by having a greater drop in viscosity during the measurements.

The other reason for procuring a gelatine preparation with a high intrinsic viscosity is the following. As already mentioned, it is possible to use a more dilute substrate solution if the preparation has a high intrinsic viscosity than otherwise, and in determining the "Michaelis constant" we should also be able to use substrate solutions with low concentrations, at least concentrations which approach the apparent value of the "Michaelis constant".

As a result of these considerations, we decided as a first goal to find a gelatine with so much higher intrinsic viscosity than the preparations previously used extensively by one of us, that the sensitivity of the viscosimetric method for the determination of proteolytic activity thereby would be expected to increase ten times.

The intrinsic viscosity of a solution of gelatine changes very little with alterations in pH and alterations in salt concentration if pH is 4.7 (the isoelectric point) and if the sodium chloride concentration of the solution is 4.7 % (0.8 M); at this salt concentration there is a viscosity maximum [19]. Viscosimetrical determinations with gelatine are usually carried out at 35.5 °C[20]. We considered it appropriate to carry out the experiments for the assay of the intrinsic viscosities of our gelatine preparations under these conditions. It may be mentioned that other conditions have also been used, *e. g.*, by Pouradier and Venet [19]. The determinations were made as follows. Three solutions with different concentrations (for commercial preparations 4, 3, 2, and 1 %, for preparations with higher intrinsic viscosity 2.0, 1.5, 1.0, and 0.5 %) were made up, and the viscosity was measured. The calculations were carried out according to Mead and Fuoss' formula [21]

$$\frac{\ln \eta_r}{c} = [\eta] - \beta[\eta]^2 c \qquad (5)$$

where η_r is the relative viscosity, $[\eta]$ the intrinsic viscosity, c the concentration of the high polymer substance in grams per 100 ml, and β a constant which is characteristic for the high polymer substance.

Thus the natural logarithm of the relative viscosity was divided by the concentration in per cent, and the quotient was plotted in a diagram *versus* the concentration. A straight line was fitted to the points. Its intersection with the axis of ordinates gave the intrinsic viscosity.

Previously, in the viscosimetrical determination of proteolytic activity, one of us [2,3] has used a commercial preparation of granular gelatine (Fisher Sc.). This gelatine has an intrinsic viscosity of 0.21. In order to get a sample with higher intrinsic viscosity, we procured eight other commercial gelatines. The following values were obtained for their intrinsic viscosities: 0.43, 0.38, 0.38, 0.37, 0.38, 0.38, 0.36, and 0.38. We also considered these preparations to have too low intrinsic viscosity. Hence, we decided upon preparing a gela-

tine with a higher degree of polymerization by fractionated precipitation of a solution of one of the commercial gelatine preparations by ethanol according to Pouradier and Venet [19,22].

The high polymer gelatine, used in the following experiments, was prepared in the following way. 200 g of a commercial gelatine preparation with the intrinsic viscosity 0.38 (measured at 35.5 °C and pH 4.7 in a 0.8 M sodium chloride solution) were dissolved in 4 500 ml of water. At a temperature of 42 °C 5 200 ml of abs. alcohol were added, whereby a strong precipitate was formed. The mixture was heated so that the precipitate dissolved, whereupon it was left in a thermostat room at 42 °C till the following morning. Then a layer containing 51 % of the gelatine had separated out on the bottom of the container. The layer was taken out and dissolved in 1 650 ml of water. Part of this solution was precipitated at −5 °C with acetone, and the precipitate was dried *in vacuo*. The intrinsic viscosity of the preparation was 0.71. The preparations we obtained in other fractionation experiments usually showed no significant difference from this preparation. However, as a result of some successive fractionation of 20 g of gelatine we obtained 0.78 g of a preparation with the intrinsic viscosity 1.0.

Those properties of the various gelatine preparations which are of special interest in this investigation are listed in Table 1.

Table 1. The properties of some gelatine preparations.

$[\eta]$	Molecular weight	Concentration in per cent		Relative enzyme concentration for the same decline in viscosity
		$\eta_r = 2$	$\eta_r = 3$	
0.21	40 000	3.4	5.6	1
0.38	100 000	1.9	3.2	0.3
0.71	300 000	1.0	1.7	0.09
1.0	500 000	0.7	1.2	0.05

The relative molecular weights of the preparations (relative to the Fisher gelatine previously used by one of us) was calculated according to the formula of Mark [23]

$$[\eta] = K \cdot M^a \tag{6}$$

where M is the molecular weight, and K and a are constants. For gelatine, a has the value 0.885 according to Pouradier and Venet [22]. Another figure is given by Williams, Saunders, and Cicirelli [34], who — from measurements carried out under iso-electric conditions and in the presence of salt, using preparations with intrinsic viscosities between 0.137 and 0.35 — found the values $K = 2.9 \cdot 10^{-4}$ and $a = 0.62$. Using their values, we have calculated the weight average molecular weights of our preparations as given in Table 1. As it is not quite certain that their viscosity determinations were done under the same conditions as ours, and as two of our preparations have much higher intrinsic viscosities than their highest sample, the molecular weights computed for our preparations are not very accurate but give at least the right order of magnitude.

For viscosimetric determinations of enzymic activity solutions with the relative viscosity $\eta_r = 3$ are suitable. The concentration of solutions with this viscosity were read from the graphs for the calculation of the intrinsic viscosity for the various preparations. The relative enzyme concentrations, which give the same decline in viscosity for equally viscous ($\eta_r = 3$) solutions of the various gelatine preparations was computed according to equations (4) and (6); the squares of the concentrations at which solutions of the various preparations have the relative viscosity 3 were divided by the square of the concentration, at which a solution of the previously used gelatine with the intrinsic viscosity 0.21 has the relative viscosity 3.

It is evident from Table 1 that the sensitivity of the viscosimetrical method for the determination of enzymic activity is increased about ten times, if the fractionated gelatine with the intrinsic viscosity 0.71, which could easily be prepared, is substituted for the Fisher gelatine previously used. This improvement was, however, somewhat counteracted by the fact that the slow decrease in viscosity in the absence of enzyme was greater in the case of the more high molecular gelatine.

Activity determinations

The enzymic activity at various substrate concentrations was determined as follows [22-26]. Using a fractionated gelatine with the intrinsic viscosity 0.71, we made up six solutions of the concentrations 2.00, 1.75, 1.50, 1.25, 1.00, and 0.75 %, respectively, in phosphate buffer of pH 6.50 and the ionic strength 4/30 (in order to get the ionic strength 0.1 in the final reaction mixture). Merthiolate (0.01 %) was added as a preservative. The solutions were kept in a thermostat bath at 35.5 °C.

Two experimental series were carried out, using solutions of crystalline trypsin. The reaction mixtures were made up of 1 ml of enzyme solution and 3 ml of substrate solution. They were transferred to an Oswald viscosimeter which had been calibrated carefully with sugar solutions [27], and the viscosity measured at various times as usually. In calculating the specific viscosities, due allowance was made for the viscosity and density of a corresponding water solution of the buffer salts. The measurements at different substrate concentrations were made in a randomized order.

CALCULATION OF THE RESULTS

The computation of the activity from viscosimetrical measurements is usually done graphically, whereby the inverted values of the relative viscosities $1/\eta_{sp}$ are plotted *versus* time, whereupon a straight line is fitted by the eye to the points and the inclination read [18,24,27]. However, in this case we preferred to calculate the inclinations of the lines by the method of least squares. The inclinations were multiplied by the squares of the substrate concentrations in the reaction mixtures according to eqn. (4) [18].

The activity v was expressed [18,28] in $m\mu A$ ($A \cdot 10^{-9}$). We also calculated $[S]/v$, where [S] is the substrate concentration in the reaction mixture, expressed in per cent. In a diagram, $[S]/v$ was plotted *versus* [S] [17] and a straight line was fitted to the points (Figs. 1—2). The "Michaelis constant" can — analogously to a similar suggestion by Dixon [29] — be read as the point of

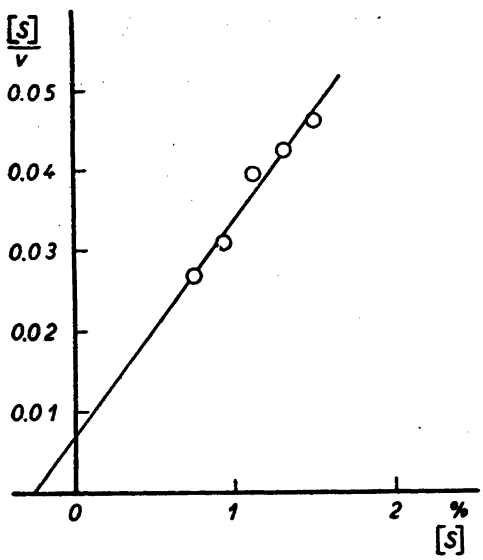

Fig. 1.

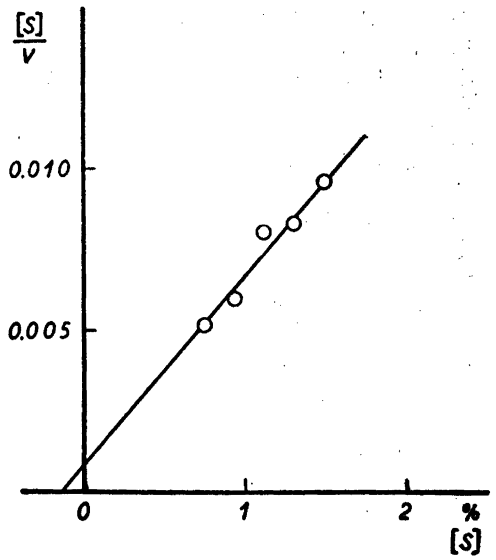

Fig. 2.

Graphs for the calculation of the "Michaelis constant" for trypsin. The value of the constant — however, with negative sign — is read at the intersection between the abscissa and the fitted line.

intersection — however, with positive sign — between the fitted line and the abscissa *.

DISCUSSION

We can see from Figs. 1—2 that all reaction mixtures used for the activity determinations, had a higher substrate concentration than corresponds to the "Michaelis constant". It turned out that the accuracy of the activity determinations at the actual substrate concentration 0.56 % (using the 0.75 % stock solution) was so low that the determinations could not be taken into consideration. The relative viscosity of the actual reaction mixtures was only 1.4, and even an error of only 0.2 seconds in the reading of a flow time had a marked influence on the result. Hence, the measurements with the 0.75 % stock solution of gelatine were not considered in the calculations. The values of the Michaelis constant as read from Figs. 1 and 2 are 0.03 and 0.01 % respectively.

* A linear relationship for the calculation of "Michaelis constant" can also be obtained in two other ways, but this way not only makes it possible to read the value of the constant directly in a graph but also has the advantage of being more suitable than the other for numerical calculation of Michaelis constant by the method of least squares. This depends on the fact that for such a calculation one variable should be actually independent, in this case [S], and that the accuracy of the other variable, in this case [S]/v, should be independent of the value of the first variable. The first condition is met in this case, and also if we plot $1/v$ *versus* 1/[S], but not if we plot v *versus* v/[S]; however, the second condition is not met with if we plot $1/v$ *versus* 1/[S] but is approximately met with if we plot [S]/v *versus* [S].

It is evident that the accuracy in the determination of the constant is rather low as a consequence of the considerable extrapolation. It would have been desirable to have measurements at lower substrate concentrations; however, for such measurements it would have been necessary to use a gelatine with higher intrinsic viscosity than 0.7.

It is of interest to compare the value of "Michaelis constant" obtained by this viscosimetrical method to values obtained by other methods. Northrop [30] measured the activity of trypsin on gelatine solutions at pH 6.0 using a conductometric method. For one experimental series (*loc. cit.*[30] Table V) he gives the value 0.5 % for the "Michaelis constant". From another experimental series (*loc. cit.*[30] Table VI) we have calculated the value 0.3 %.

Northrop [31] also investigated the influence of trypsin on gelatine solutions of different concentrations by measuring the viscosity at various times. As the change of viscosity at the breakdown of gelatine proceeds differently at the beginning than later on — probably [24] as a consequence of the various types of amino acid linkages which are not split with equal ease — and as Northrop made only a few measurements, however, over a wide interval, it is not possible to calculate the "Michaelis constant" from his experiments.

We can conclude from the experiments that it is possible to determine the "Michaelis constant" of proteolytic enzymes viscosimetrically using gelatine as substrate. However, with a gelatine with the intrinsic viscosity 0.7 (as measured at 35.5 °C and pH 4.7 in 0.8 M sodium chloride solution) the sensitivity of the method permits only measuring of constants with higher value than about 0.1 %. The sensitivity can, however, be increased somewhat by using a gelatine with higher intrinsic viscosity than 0.7. As already mentioned in this paper a gelatine with the intrinsic viscosity 1.0 can be prepared from commercial gelatine by a few fractionated precipitations with ethyl alcohol at 42 °C according to Pouradier [19]. It is evident that it would be highly desirable to use such a gelatine preparation for the reaction mixtures with the lowest substrate concentrations, *e. g.*, about 0.5 %, in order to increase the accuracy The accuracy of the determinations can, of course, be increased also by increasing the number of experiments in each series.

It should finally be mentioned that in reaction mechanism determinations when viscosimetric activity measurements are carried out at different pH values, it is necessary to correct the values for the influence of pH on the viscosity of the substrate [32], and that it may be desirable in calculating the activities to use the more accurate formula [32,33] $A = J\, c_s^2\, (\mathrm{d}\ 1/\ln\ \eta_r)/\mathrm{d}t$ instead of the formula given here as eqn. (4). However, the accuracy of the determination of the "Michaelis constant" must not necessarily be very high, as the value is usually independent of the pH in some region (the true value of the constant) and changes very much with the pH in some other region (apparent values of the "Michaelis constant"); the logarithm of the factor by which the constant changes if pH is changed by one unit gives the number of hydrogen ions which take part in the proton exchange necessary for the combination of the enzyme and substrate.

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Solubility Properties of Non-ionic Water-soluble Cellulose Ethers in Mixtures of Water and Alcohol *

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The behaviour of some non-ionic water-soluble cellulose ethers in mixtures of water and methanol, ethanol, *n*-propanol, *iso*propanol, *tert*-butanol or acetone was investigated by means of viscosity and turbidity measurements. A viscosity minimum and corresponding turbidity maximum were found for ethyl cellulose and ethyl-hydroxyethyl cellulose in some of the solvent mixtures. These effects are due to variations of the solubility of the cellulose ether. The well-known tendency of water and alcohol molecules to associate provides a possible explanation of the effect. The viscosity at 20° C of mixtures water-acetone, water-*iso*propanol and water-*tert*-butanol was determined.

Non-ionic cellulose ethers, soluble in water, are synthesized by introducing into the glucose residues of the cellulose chains moderate amounts of different substituents such as methyl, ethyl or hydroxyethyl groups, either one kind only or combined with each other. These cellulose ethers are insoluble in alcohols although they swell strongly. A peculiar phenomenon, which seemed to deserve further studies, was observed during experiments to investigate the solubility of ethyl-hydroxyethyl cellulose in water-alcohol mixtures. In some cases the solubility passes through a pronounced minimum with increasing alcohol content. The effect has been mentioned in a review article [1].

MATERIALS INVESTIGATED

Commercial cellulose ethers:

Methyl cellulose (Methocel [2] from Dow Chem. Corp., USA).
Methyl-hydroxyethyl cellulose (Glutolin from Kalle und Co. AG., Germany).
Ethyl-hydroxyethyl cellulose (Modocoll [3] from Mo och Domsjö AB, Sweden).

* Part of this paper was presented at the XIIIth International Congress of Pure and Applied Chemistry, Stockholm, July and August 1953.

The physico-chemical properties of water-soluble ethyl-hydroxyethyl cellulose have been described by Jullander [4,5]. It should not be identified with ethyl-hydroxyethyl cellulose recently brought on the market by Hercules Powder Co under the name EHEC, and which is insoluble in water but soluble in a number of organic solvents [6,7].

Cellulose ethers made in the laboratory:

Ethyl cellulose was prepared from alkali cellulose through etherification with ethyl chloride. With increasing degree of substitution, the solubility of ethyl cellulose changes from solubility only in alkali over solubility in water to solubility in organic solvents. The available information as to the range of water solubility is scarce as well as confusing and a special investigation was made to settle this point [8]. Hydroxyethyl cellulose was obtained from alkali cellulose by etherification with ethylene oxide dissolved in ethyl chloride. If the temperature is kept sufficiently low, the ethyl chloride only acts as an inert diluent, *cf.* Sönnerskog [9].

Ethyl cellulose: the sample used was prepared in the same way as described in the investigation quoted. Raw material: sulphite cellulose from spruce, alpha content 89.7 % (CCA 7), cuprammonium viscosity (TAPPI) 22 cp, ethanol extract 0.48 %. Steeping lye 33.0 %, mercerization 45 min. at 24° C, press factor 2.58. Reaction time 210 min. at 100° C.

Hydroxyethyl cellulose: the same pulp as above was used as raw material. Steeping lye 18.7 %, mercerization 45 min. at 25° C, press factor 2.14. Ethylene oxide 2.75 moles per glucose unit of the cellulose, was dissolved in ethyl chloride, reaction 5 hours at 38°—43° C. The raw product obtained was neutralized with CO_2, and washed with 95 % ethanol acidified with acetic acid.

Some properties of the ethers are recorded in Table 1.

Table 1. Summary of properties of cellulose ethers.

Cellulose ether	Substituent	Degree of * substitution (DS)	Analytical methods Ref. No.	Viscosity ** cp
Methyl cellulose (Methocel 400)	Methyl	1.97	10	310
Methyl-hydroxyethyl cellulose (Glutolin Leim)	Methyl	1.11	11, 12	140
	Hydroxyethyl	0.14		
Ethyl-hydroxyethyl cellulose A (Modocoll M, batch No. 08822)	Ethyl	1.34	12, 13	65
	Hydroxyethyl	0.44		
Ethyl-hydroxyethyl cellulose B (Modocoll E 100, batch No. 34011)	Ethyl	0.74	12, 13	90
	Hydroxyethyl	0.65		
Ethyl cellulose	Ethyl	1.20	10	—
Hydroxyethyl cellulose	Hydroxyethyl	1.31	12	—

* Calculated on bone-dry and salt-free basis.

** Water solution at 20° C, 2 % airdry cellulose ether. Höppler viscometer, precision model.

EXPERIMENTS

Solutions of cellulose ethers in water are never molecularly dispersed to 100 %. Fragments of fibers and gel particles are always present to some extent.

Viscosity and turbidity are important physical characteristics of cellulose ether solutions and were therefore determined. A comparison of measurements in water/alcohol and water only can also be used to estimate the molecularly dispersed portion of the cellulose ether. It is here assumed that a decreased viscosity and increased turbidity indicate a decreased "solubility", *i.e.* a smaller portion of the material is molecularly dispersed. The reverse may not always be true. A notable example from the chemistry of cellulose derivatives is the viscosity increase during the ripening of viscose. The turbidity does not change in this process.

The viscosity was measured at 20° C with a Brookfield viscometer (type LVF, usually spindle 1 at 30 and 60 rpm) on a system containing 1 wt. % of cellulose ether and 99 wt. % of a mixture of water and alcohol. Turbidity measurements were made on the same solutions by means of a method described earlier [4,14,15]. The results are expressed as % transmission, 100 % meaning that no measurable amount of light is scattered. The transmission values are not affected by light absorption.

The instrument was a Lumetron photoelectric colorimeter, Model 402-E (Photovolt Corp., New York, USA). Green filter (M 550); light path in cuvette 20 mm; displacement 12 cm.

A typical example of the effect obtained is shown in Fig. 1 where solutions of ethyl-hydroxyethyl cellulose A in mixtures of water and *n*-propanol were

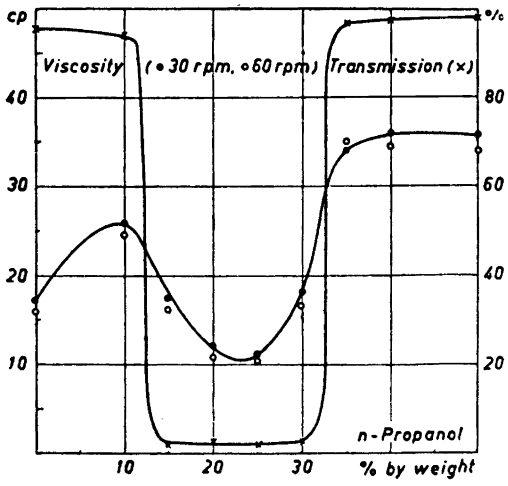

Fig. 1. Viscosity and transmission of solutions of 1 % ethyl-hydroxyethyl cellulose A in mixtures of water and n-propanol. Temp. 20° C.

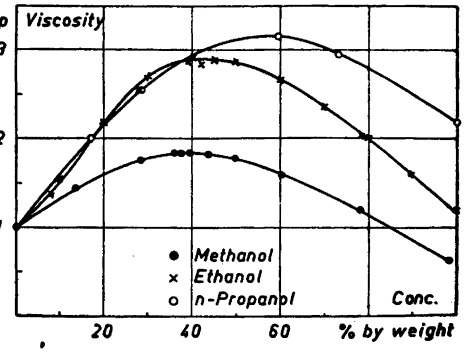

Fig. 2. Viscosity of mixtures of water and methanol, ethanol or n-propanol. Temp. 20° C.

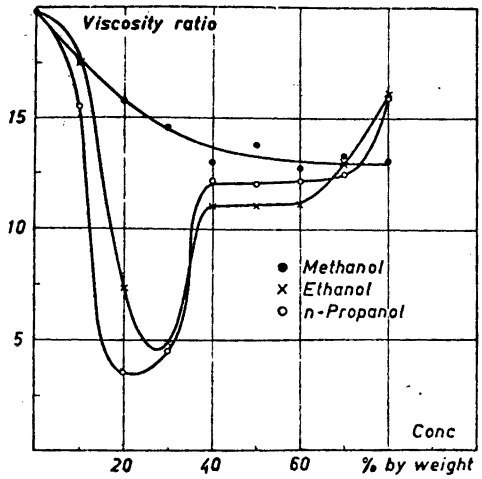

Fig. 3. Viscosity ratio of solutions of 1 % ethyl-hydroxyethyl cellulose A in mixtures of water and methanol, ethanol or n-propanol. Temp. 20° C.

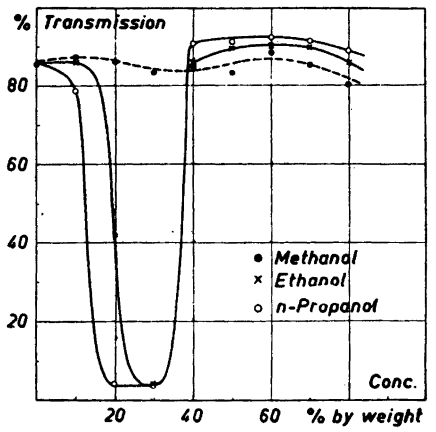

Fig. 4. Transmission of same solutions as in Fig. 3.

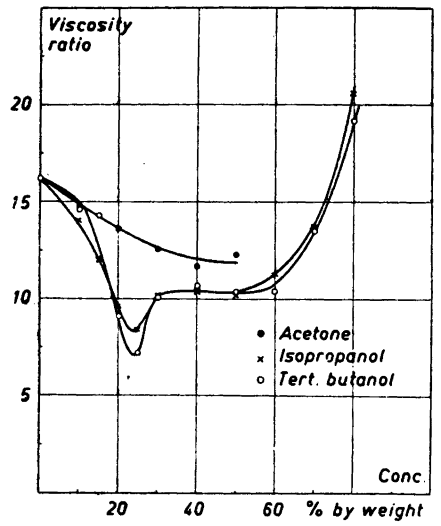

Fig. 5. Viscosity ratio of solutions of 1 % ethyl-hydroxyethyl cellulose A in mixtures of water and acetone, isopropanol or tertiary butanol. Temp. 20° C.

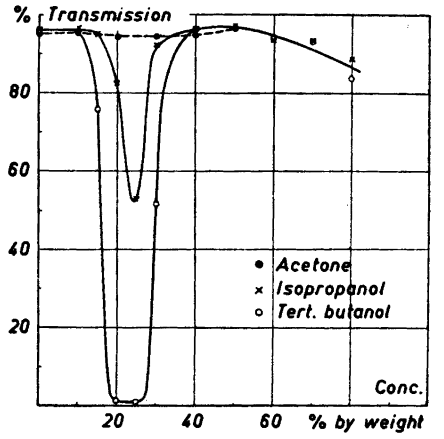

Fig. 6. Transmission of same solutions as in Fig. 5.

studied. The decrease in viscosity around the alcohol concentration 20 % by weight is counteracted by the well-known fact that the viscosity of mixtures of alcohol and water passes through a pronounced maximum when the composition is varied (Fig. 2). The values plotted were taken from the tables of Landolt-Börnstein [16]. It is therefore more correct to plot instead the viscosity ratio, η/η_0, against the solvent composition.

Fig. 1 shows that the structural viscosity, as expressed by the difference in readings of the Brookfield viscometer at 30 and 60 rpm resp., is moderate and of the same magnitude in water as in water-alcohol mixtures. Only occasionally does the difference exceed 10 %. The same was generally found in the measurements which are presented below but to avoid complicating the diagrams only measurements at 30 rpm are given. It is concluded, that in those cases where a viscosity maximum at 50—60 % alcohol concentration is found, this maximum is not due to gel formation.

Figs. 3 and 4 show viscosity ratio and transmission for solutions of ethylhydroxyethyl cellulose A within the concentration range 0—80 % of methanol, ethanol and *n*-propanol. The influence of the two remaining alcohols, which are completely miscible with water, was also investigated together with acetone (Figs. 5 and 6). No data for the viscosity of mixtures of water with *iso*propanol, *tert*-butanol or acetone usable for the calculation of viscosity ratios at 20° C were found in the literature. The viscosity of these mixtures was therefore measured with an Ostwald viscometer (Table 2 and Fig. 7).

Data for viscometers (No. 8 used for acetone, No. 14 used for *iso*propanol and *tert*-butanol):

	No. 8	No. 14
Volume of bulb:	1.25 ml	0.79 ml
Length of capillary:	9.9 cm	10.1 cm
Time for outflow of water, 20° C	133 sec	62 sec

Density determinations with precision areometer.

Table 2. Viscosity at 20° C of mixtures tertiary butanol-water, isopropanol-water and acetone-water.

Organic solvent % by weight	Viscosity, cp		
	Butanol	*i*-Propanol	Acetone
0	1.00	1.00	1.00
10	1.75	1.62	1.24
20	2.84	2.49	1.45
30	3.85	3.18	1.56
40	4.78	3.57	1.54
50	5.50	3.73	1.40
60	6.01	3.67	1.17
70	6.23	3.41	0.91
80	6.07	3.02	0.65
90	5.62	2.58	0.45
100	—	2.38	0.31

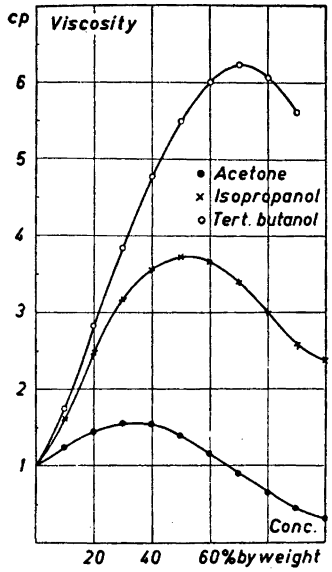

Fig. 7. Viscosity of mixtures of water and acetone, isopropanol or tertiary butanol. Temp. 20° C.

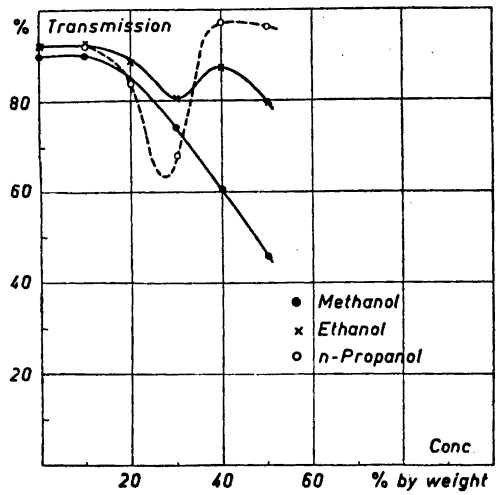

Fig. 8. Transmission of solutions of 1 % ethyl-hydroxyethyl cellulose B in mixtures of water and methanol, ethanol or n-propanol. Temp. 20° C.

The viscosity ratios obtained were corrected for the kinetic energy error, see Schulz [17]. The correction never exceeds 0.5 % except for the highest acetone concentrations.

Material: tertiary butanol (Eastman Kodak Co), *iso*propanol (purum) and acetone (puriss.) were all fractionated in a column with about 20 theoretical plates and the middle fractions collected. Boiling point interval $\leq 0.2°$ C. Density (with areometer): acetone 0.791; *iso*propanol 0.787.

From the diagrams presented so far it is found that mixtures of methanol and acetone with water do not show any effect at all. Mixtures of water with ethanol, *n*-propanol and *tert*-butanol give a strong effect, *iso*propanol a moderate effect.

Further experiments were carried out with other cellulose ethers: ethyl-hydroxyethyl cellulose B (Fig. 8), methyl cellulose (Fig. 9), methyl-hydroxyethyl cellulose (Fig. 10). Samples of ethyl cellulose and hydroxyethyl cellulose were also investigated (Fig. 11).

The phenomenon is very sensitive to the chemical composition of the cellulose ether. Even the comparatively small difference between ethyl-hydroxyethyl cellulose A and B results in effects of quite different magnitude. The transmission minimum for ethanol in Fig. 8 seems to be real even if it is small, it has been observed repeatedly. Methyl cellulose and methyl-hydroxyethyl cellulose do not show any insolubility region. Fig. 11 is interesting. The decrease in transmission and viscosity for ethyl cellulose at very low alcohol concentrations is displaced towards higher values by hydroxyethyl groups.

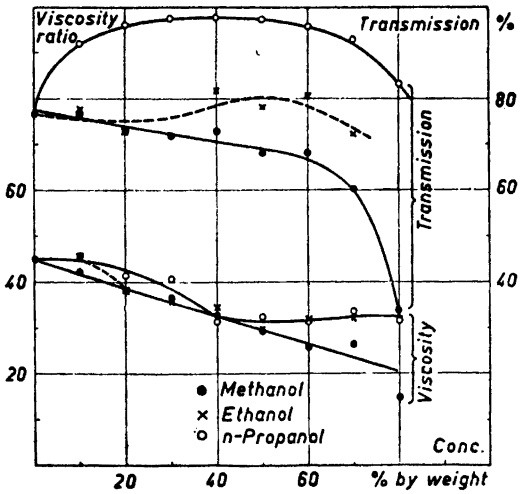

Fig. 9. Viscosity ratio and transmission of 1 % solutions of methyl cellulose in mixtures of water and methanol, ethanol or n-propanol. Temp. 20° C.

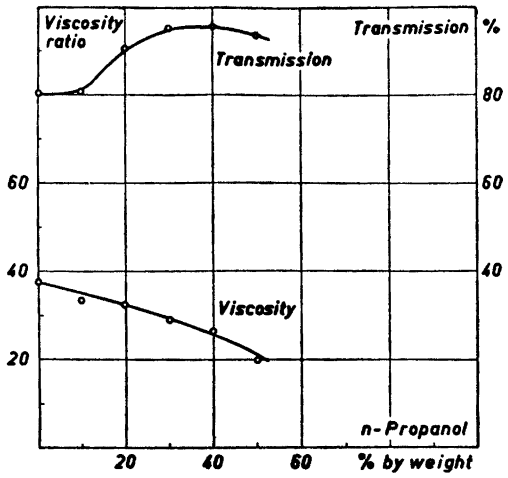

Fig. 10. Viscosity ratio and transmission of 1 % solutions of methyl-hydroxyethyl cellulose in mixtures of water and n-propanol. Temp. 20° C.

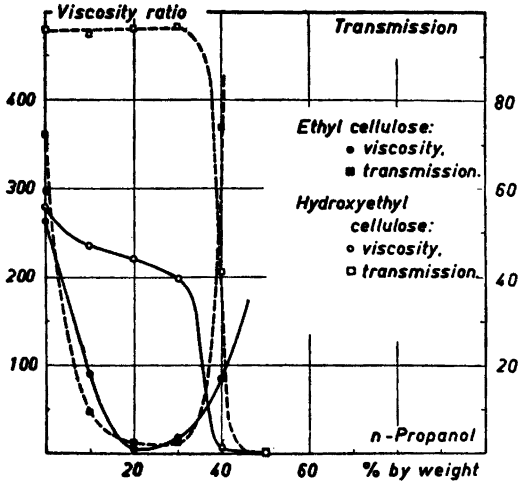

Fig. 11. Viscosity ratio and transmission of 1 % solutions of ethyl cellulose and hydroxyethyl cellulose in mixtures of water and n-propanol. Uemp. 20° C.

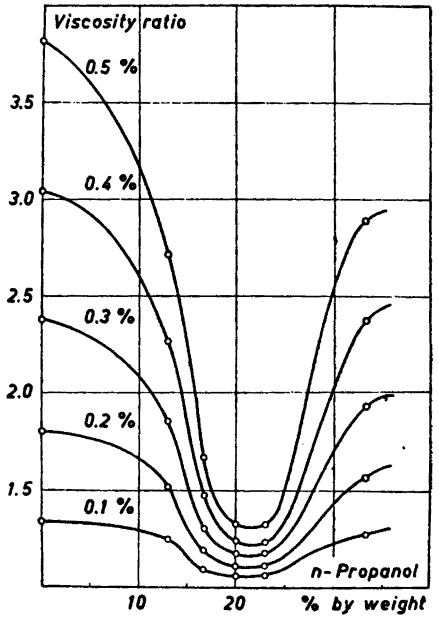

Fig. 12. Viscosity ratio of dilute solutions of ethyl-hydroxyethyl cellulose A in mixtures of water and n-propanol.

Preparation of solutions:

(Figs. 3, 4 and 9.) Stock solutions in pure water were made by swelling 5 g cellulose ether with 145 g boiling water, adding water of 20° C as required and cooling the solution to 20° C. Organic solvent was then added and the solution left in a thermostat (20° C) at least over night. In the solutions with 80 % alcohol, less water had to be used. The solutions were neither frozen, filtered, nor centrifuged.

(Figs. 1, 5, 6, 8, 10, 11.) A 2 % stock solution of cellulose ether in water was prepared, frozen, thawed and then centrifuged — repeatedly if necessary — in order to get as clear solutions as possible. To the solution was then added water and organic solvent as required. The ethyl cellulose was dissolved in 2 % sodium hydroxide, the solution neutralized with hydrochloric acid and dialysed.

The composition of the solvent mixture is always expressed as parts by weight of alcohol per 100 parts of alcohol + water. The cellulose ether concentration is expressed as g/100 g solution.

All measurements hitherto described were made on solutions of 1 % concentration with respect to the cellulose ether content. It seemed to be of interest to study the effect also at lower concentrations in a typical case. The system ethyl-hydroxyethyl cellulose A in water — *n*-propanol was chosen for that purpose.

The effect is quite noticeable also at lower concentrations, as is seen from Fig. 12 where viscosity curves in the region 0—35 % *n*-propanol are drawn for cellulose ether concentrations from 0.5 % downwards. The result is another proof that the cellulose ether is molecularly dispersed on both sides of the minimum. If the high viscosity previously found in solutions rich in alcohol were due to some sort of gel structure, the latter would break down upon dilution.

Corresponding values of the limiting viscosity number, $[\eta]$, are given in Fig. 13. These values were determined by extrapolation of η_{sp}/c versus c as usual. Often but not always the curve had a tendency to an upward bend near zero concentration; examples are given in Fig. 14. Extrapolation of the

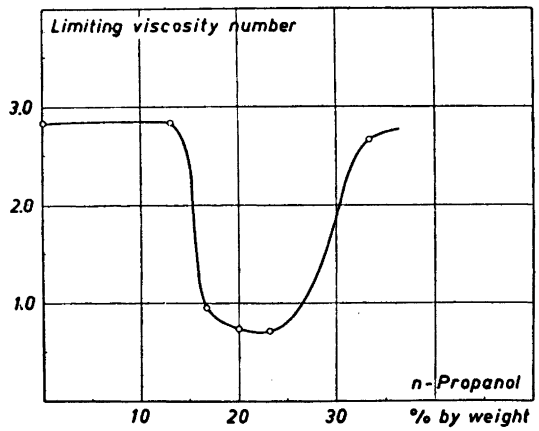

Fig. 13. Limiting viscosity number, $[\eta]$, of ethyl-hydroxyethyl cellulose A in mixtures of water and n-propanol.

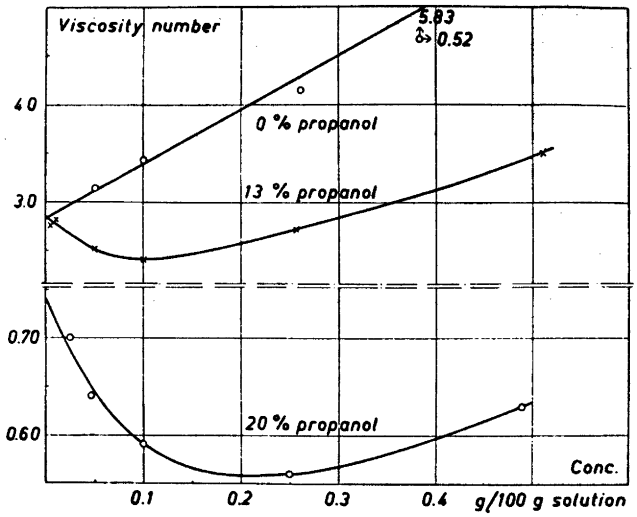

Fig. 14. Extrapolation of viscosity number, η_{sp}/c, of solutions of ethyl-hydroxyethyl cellulose A in mixtures of water and n-propanol.

viscosity number without regard to this upward bend did not very much change the general form of the curve in Fig. 13. The position of the minimum remains unchanged.

A probable explanation of this unusual behaviour upon dilution of a polymer solution is dissociation of aggregates of molecules, with increased viscosity as a consequence. A related phenomenon has been reported by Steurer [18]. The reduced osmotic pressure of ethyl cellulose (51 % OC_2H_5) dissolved in toluene showed a tendency to increase at the lowest concentrations. Steurer explained this as being due to dissociation of aggregates of molecules.

Stock solutions of ethyl-hydroxyethyl cellulose A in pure water were prepared, frozen, thawed and centrifuged mainly as already described. The required amount of *n*-propanol was then added. The exact concentration of the strongest solution, around 0.5 %, was determined through evaporation of an aliquot portion and the necessary dilutions made from this solution. The viscosity was measured at 20° C in an Ostwald capillary viscometer: time of outflow for water 123 sec., volume of bulb 0.90 ml, length of capillary 99 mm, diam. of capillary 0.402 mm. Average gradient for water 765 cm²/sec.

The concentration is expressed as g/100 g solution, not g/100 ml solution. This was done in order to avoid possible confusion arising from the contraction of the liquid mixtures studied.

As was mentioned before, the effect is very sensitive to changes in composition of the cellulose ether. It was therefore of interest to see whether the chemical composition of the precipitate which is formed at the turbidity maximum differs from that of the ether still in solution. A 0.5 % solution of ethyl-hydroxyethyl cellulose A in 20 % of *n*-propanol was prepared as described above. It was allowed to stand at 20.0° C for three days; by that time 75 %

of the cellulose ether had precipitated. The phases were separated by means of decantation and centrifugation, evaporated and dried. The chemical analysis only showed a small difference between original cellulose ether, the soluble and the insoluble parts (Table 3). Limiting viscosity numbers determined on water solutions of the fractions showed large differences and evidently the main difference between the fractions is the chain length.

Table 3. Fractionation of 0.5 % solution of ethyl-hydroxyethyl cellulose A in 20 % n-propanol.

Analysis	Unfractionated material	Precipitate	Remaining in solution
Ethoxyl groups, DS	1.34	1.31	1.20
Hydroxyethyl groups, DS	0.44	0.35	0.45
Limiting viscosity number	2.83	3.07	0.90

DISCUSSION

The experimental results will be discussed from two view-points: similar phenomena earlier published and an effort to connect the observed effect with other physico-chemical properties of alcohol-water mixtures as a means of finding at least a qualitative explanation.

The effect described in this paper, should not be mistaken for the viscosity minimum often observed when a cellulose derivative is dissolved in a mixture of liquids, neither of which is itself a solvent for the derivative. A typical example is the dissolution of nitrocellulose in ethyl ether and ethyl alcohol, a pronounced minimum viscosity is found at approximately 50 % alcohol varying with the nitrogen content [19,20]. On both sides of this point the viscosity rises sharply, ending in semisolid gel-like structures.

Similar viscosity minima have also been observed in solutions of ethyl cellulose and benzyl cellulose [21,22]; *cf.* also the discussion by Mardles [23] of solvents for cellulose esters. When cellulose nitrate or cellulose acetate is dissolved in acetone and other solvents, the viscosity passes through a minimum upon addition of small amounts of water [20,24-27]. This addition is considered as improving the solubility.

A phenomenon more similar to the effect studied here is the salting-out of proteins in the presence of *n*-propanol which has been described by Jirgensons in several publications [28-32]. In some cases it was observed that the flocculation tendency of the protein upon addition of propanol first passed through a maximum and then through a minimum. Methanol did not show this effect and *n*-propanol had a stronger effect than ethanol. Also the investigation of Verstraete [33] on the solubility of saponin in water-alcohol should be mentioned. A minimum in solubility followed by a maximum (with increasing amount of organic solvent) was observed for methanol, ethanol, *iso*propanol and acetone but *not* for *n*-propanol. Bungenberg de Jong [34,35] found that when tannin or resorcinol is added in increasing amounts to solutions of agar or gelatin, the viscosity passes through a minimum and the turbidity simultaneously through a maximum.

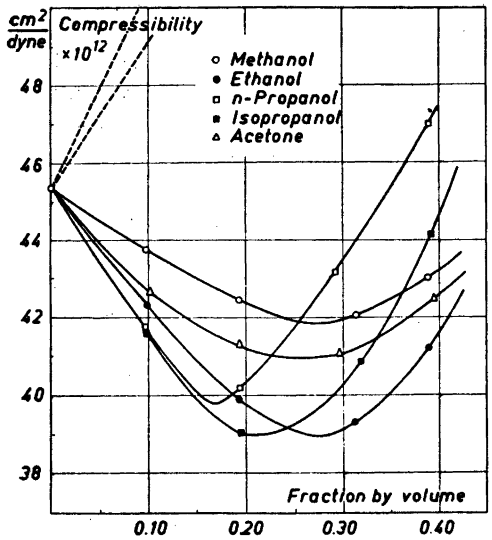

Fig. 15. Compressibility of mixtures alcohol-water and acetone-water as function of concentration of organic solvent. Temp. 20° C. Data from Jacobson (46). An ideal mixture gives a straight line within the sector indicated by the broken lines in the upper left corner.

It would be of interest to try to connect the observed solubility effects with the many abnormal physico-chemical properties of water-alcohol mixtures which are well-known in the literature; for a discussion see Kortüm [36]. They are interpreted as caused by the formation of more or less stable aggregates of water and alcohol molecules. Among the effects studied are the temperature coefficient of surface tension [37,38] and the diffusion [39–41]. Of greater interest are perhaps ultrasonic absorption and velocity, compressibility, contraction and viscosity.

The behaviour of water-alcohol mixtures in ultrasonic fields has been investigated by Burton [42] and Storey [43]; see also Refs. [44,45]. When the sound absorption is plotted against alcohol-water composition maxima are found, the magnitude of which increase strongly in the order ethanol, *iso*propanol, *n*-propanol, *tert*-butanol. No maximum was observed in mixtures of methanol and water.

Jacobson [46] studied the compressibility of alcohol-water mixtures. Part of his tabulated data are plotted in Fig. 15. The pure organic solvents have compressibilities between 83 and 99 × 10^{-12} cm²/dyne. The deviations from the ideal mixture are great in all cases, but the minima are more pronounced for ethanol and both propanols than for methanol and acetone. Position of the minima:

	Fraction by volume	% by weight
Ethanol	0.28	23
n-Propanol	0.17	14
*Iso*propanol	0.21	17

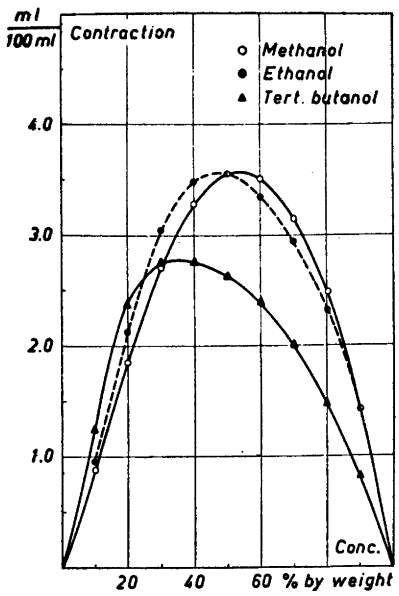

Fig. 16. Contraction of mixtures alcohol-water Temp. 20° C. Calculated from density data available in the literature.

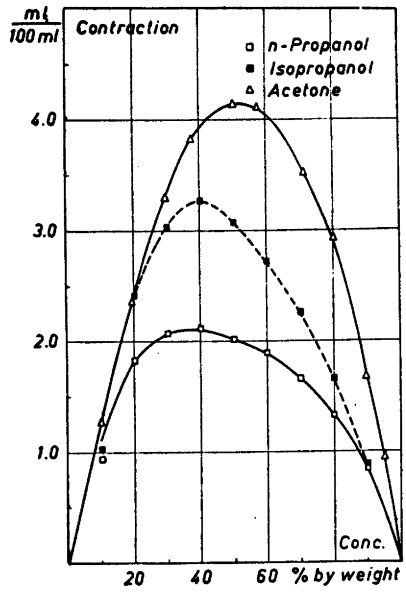

Fig. 17. See Fig. 16.

Contraction is a more direct measure of the association. Figs. 16 and 17 give the variation of contraction with the composition of the mixtures.

The contraction was calculated as recommended by Carr and Riddick [47], using what seemed to be the best available density data, taken from Ref.[48] (methanol, ethanol, *n*-propanol and *iso*propanol) and Ref.[49] (*tert*-butanol and acetone). The contraction is expressed as ml per 100 ml of constituents (not mixed). Values for *n*-propanol at 20° C were interpolated from available data for 15° and 30° C. The density of pure tertiary butanol in liquid state at 20° C was found to be 0.7850 through extrapolation. The melting point of the substance is 25.5° C.

Contrary to our expectations there is no correlation between the magnitude of the contraction and the effect studied in this paper. Acetone has the highest contraction and the curves for ethanol and methanol are close together. Position of maxima:

		corresponding to the approximate formula
Ethanol	47 %	C_2H_5OH, $3H_2O$
n-Propanol	37 %	C_3H_7OH, $6H_2O$
*Iso*propanol	41 %	C_3H_7OH, $5H_2O$
Tert-butanol	35 %	C_4H_9OH, $8H_2O$

The viscosity also passes through a maximum as has already been demonstrated (Figs. 2 and 7). Positions of the maximum deviation from a straight line relationship are:

Ethanol	42 %
n-Propanol	50 %
*Iso*propanol	45 %
Tert-butanol	60 %

Summing up, a partial correlation has been found between the solubility effects studied here, and the anomalous behaviour of water-alcohol mixtures with respect to sound absorption, compressibility, contraction and viscosity.

We believe that the following is a reasonable qualitative explanation. Water is a poor solvent for low substituted ethyl cellulose [8]. When therefore for instance propanol is added to a solution in pure water, the cellulose ether chains are easily dehydrated, and the solubility drops already at very low alcohol concentrations (Fig. 11). On the other hand the degree of substitution is too low to make the ether soluble in pure alcohol. Hydroxyethylation of cellulose makes the chains more hydrophilic and a higher alcohol concentration, 30—40 %, is needed to reach the precipitation point. In the case of ethyl-hydroxyethyl cellulose A in solutions with increasing alcohol content, the decrease in solubility at around 15 % alcohol is due to dehydration of the chains and, as expected, the precipitation point lies between those found for ethyl cellulose and for hydroxyethyl cellulose. When the alcohol concentration approaches contraction maximum, fairly strong propanol-water aggregates are formed. It must be assumed that aggregates with an average composition corresponding to the contraction maximum or slightly richer in alcohol, constitute an excellent solvent for ethyl-hydroxyethyl cellulose A. The aggregates can solvate both ethyl and hydroxyethyl groups at the same time on account of their composition. Steric effects could also play a part as obviously these »solvent molecules» are bulky and will keep the cellulose chains effectively apart. At high alcohol concentration finally, the hydroxyl groups are desolvated, and the chain can no longer remain in solution.

It is easy to understand that small changes in the composition of the ether have a large effect upon the solubility behaviour. Obviously a nice balance between ethyl groups and hydroxyl plus hydroxyethyl groups is needed to allow the alcohol-water aggregates to function properly as solvating agents. The aggregates formed between other alcohols and water may be more or less well suited for solvation of the cellulose ether and the strength of the association, as measured by contraction or some other physical property, gives no guarantee that the aggregates will have good solvating properties.

It should be noted that regardless of the size of the alcohol, the minimum solubility is found at around the same *weight* composition (25 % alcohol) and not *molar* composition of the solvent mixture.

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The Dissociation Constants of Dihydroxytartaric and Tartronic Acids

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The first and the second dissociation constant of dihydroxytartaric acid at 25° C, and the first and the second dissociation constant of tartronic acid at 25 and 37° C, have been determined by measuring hydrogen ion concentrations by means of the glass electrode in solutions containing various concentrations of potassium chloride. Formulae expressing the dissociation constants as functions of the ionic strength of the solutions are given. The activity dissociation constants have been estimated by extrapolation to zero ionic strength.

Dihydroxytartaric acid decomposes in aqueous solution into tartronic acid and carbon dioxide. In a kinetic study of this reaction reported in the following paper [1], it was of importance to know the dissociation constants of the two organic acids. They were therefore determined by means of the glass electrode in solutions containing potassium chloride. While the two dissociation constants of tartronic acid were determined both at 25 and 37° C, those of dihydroxytartaric acid were, owing to the too rapid decarboxylation at 37°, only determined at 25° C.

The sparingly soluble di-sodium salt of dihydroxytartaric acid was prepared by the method of Filippo [2]. It was purified in the following way. The crude salt was dissolved in twice the equivalent amount of 2 M hydrochloric acid at about 5° C. The solution was filtered and added an excess of 2 M sodium acetate. The precipitate was isolated by suction and washed with a large amount of redistilled water. Since it has been found by Lachman [3] that the salt has an enormous tendency to occlude other salts, the washing was continued long after the filtrates failed to give an opalescence with silver nitrate. The crystals were dried in the air and, finally, in vacuum over concentrated sulphuric acid. When samples of the dry product were ignited in a platinum crucible, the residue dissolved in hydrochloric acid, and the solution titrated back with sodium hydroxide, a molecular weight of 263.1 was found, while 262.1 is calculated for the formula $[C(OH)_2COONa]_2$, $2H_2O$. The purified preparation contained less than 0.05 % sodium chloride as shown by Volhard titration.

Tartronate buffers were prepared by complete decarboxylation of solutions of dihydroxytartrate. The other chemicals used were of analytical purity. Redistilled water was used to make up the solutions.

The measurements were carried out and the hydrogen ion concentrations computed by the same procedure as that followed by the author in an earlier paper [4]. In the following tables, E denotes the e.m.f. in volts (measured by means of the glass electrode) of the cell

$$H_2;\ X;\ 3.5\ M\ KCl;\ S;\ H_2$$

where X denotes the solution under investigation, while S is always the solution: 0.00999 molar HCl, 0.0900 molar NaCl. The composition of solution X may be expressed as

$$a \text{ molar HCl, } b \text{ molar } Na_2T, \ s \text{ molar KCl}$$

where T denotes either dihydroxytartrate or tartronate.

THE DISSOCIATION CONSTANTS OF DIHYDROXYTARTARIC ACID

Dihydroxytartrate buffers were prepared by weighing out the sodium salt and dissolving it in a solution containing appropriate amounts of hydrochloric acid and potassium chloride. The cell was made up, and the measurements were started as quickly as possible, in general about 8 minutes after the salt was dissolved. Readings were taken with short intervals for about half an hour. Owing to decarboxylation, E increased gradually with a rate which varied with the composition of the solution examined (from 0.02 to 0.15 millivolts per minute). The values of E given in Table 1 were found by extrapolation of the readings to the time when the salt was dissolved.

Three series of solutions were measured; within each the ionic strength I was nearly constant. The results are shown in Table 1 where the hydrogen ion concentrations h obtained directly from the measurements are given in the next to the last column. The first and the second dissociation constant of dihydroxytartaric acid, K_1 and K_2, respectively, were computed from the data in the following way. From the two equations

$$[H_2T] + [HT^-] + [T^{--}] = b$$

and

$$2[H_2T] + [HT^-] = a - h$$

and the usual expressions for K_1 and K_2, we derive the equation

$$\frac{(2b-a+h)\ h^2}{a-h} = K_1K_2 + \frac{(a-b-h)\ h}{a-h}\ K_1 \qquad (1)$$

As a first approximation, we neglect the small variation of K_1 and K_2 within a series. By means of eqn. (1) we compute graphically the constants K_1 and K_1K_2 for each of the three series. A correction for the influence of the small variation in ionic strength within a series was made in the following way. Eqn. (1) was written as

$$\frac{(2b-a+h)\ h^2}{a-h}\ f_1f_2 = K_{1m}K_{2m} + \frac{(a-b-h)h}{a-h} f_2\ K_{1m} \qquad (2)$$

Table 1. Measurements of hydrogen ion concentrations in dihydroxytartrate buffers at 25° C.

a	*b*	*s*	*I*	*E*	−log *h* (found)	−log *h* (calc.)
0.02999	0.01502	0.0700	0.1106	−0.0002	1.985	1.986
0.02744	0.01499	0.0700	0.1089	0.0048	2.070	2.070
0.02000	0.01000	0.0800	0.1079	0.0077	2.118	2.117
0.01499	0.01002	0.0800	0.1046	0.0243	2.400	2.397
0.00999	0.00498	0.0900	0.1047	0.02175	2.356	2.354
0.00747	0.00505	0.0900	0.1030	0.0368	2.609	2.613
0.00499	0.00497	0.0900	0.1021	0.0605	3.011	3.011
0.03001	0.01502	0.1700	0.2110	−0.0005	1.972	1.972
0.02747	0.01505	0.1700	0.2093	0.0042	2.052	2.055
0.01998	0.01000	0.1800	0.2082	0.0075	2.107	2.105
0.01499	0.01000	0.1800	0.2049	0.0233	2.375	2.375
0.00999	0.00498	0.1900	0.2048	0.02155	2.344	2.342
0.00747	0.00502	0.1900	0.2031	0.03595	2.589	2.591
0.00499	0.00498	0.1900	0.2023	0.05875	2.976	2.972
0.03002	0.01504	0.2700	0.3112	−0.00095	1.966	1.964
0.02744	0.01500	0.2700	0.3094	0.00395	2.049	2.045
0.02498	0.01501	0.2700	0.3080	0.0090	2.134	2.137
0.01999	0.01003	0.2800	0.3084	0.0067	2.094	2.098
0.01748	0.01003	0.2800	0.3066	0.0140	2.218	2.216
0.01499	0.01003	0.2800	0.3051	0.02265	2.364	2.364
0.01200	0.00998	0.2800	0.3038	0.03675	2.603	2.599
0.00999	0.00504	0.2900	0.3050	0.02095	2.334	2.340
0.00747	0.00508	0.2900	0.3033	0.03535	2.578	2.584
0.00499	0.00503	0.2900	0.3024	0.0576	2.957	2.950

where K_{1m} and K_{2m} denote the values of K_1 and K_2 corresponding to the average value of the ionic strength in the series (I given in Table 2), while f_1 denotes the ratio K_{1m}/K_1 and f_2 the ratio K_{2m}/K_2. The ratios f_1 and f_2, which are both very close to unity, were computed by means of the expressions (3) and (4) (or, actually, from preliminary expressions practically identical with these). Finally, the constants K_{1m} and $K_{1m}K_{2m}$ were computed graphically by means of eqn. (2). The dissociation constants thus obtained are shown in the third and fifth column of Table 2, while the corresponding ionic strengths are given in the second column. For simplicity, the subscripts m have been omitted. In order to demonstrate the agreement with the experimental data, the values found for K_1 and K_2 were introduced into eqn. (2), and h was calculated for all the solutions examined. The results obtained are given in the last column of Table 1.

Table 2. The first and the second dissociation constant of dihydroxytartaric acid at 25° C

Series	*I*	−log K_1	−log K_1 (calc.)	−log K_2	−log K_2 (calc.)
1	0.1060	1.694	1.696	3.513	3.518
2	0.2062	1.642	1.640	3.435	3.422
3	0.3063	1.612	1.613	3.378	3.385

Table 3. The first dissociation constant of tartronic acid at 25° C.

a	*b*	*s*	*I*	*E*	$-\log h$	$-\log K_1$	$-\log K_1$ (calc.)
0.02999	0.01502	0.0700	0.1076	0.0077	2.120	2.114	2.116
0.02744	0.01500	0.0700	0.1060	0.01395	2.226	2.123	2.117
0.02000	0.01000	0.0800	0.1058	0.0148	2.239	2.118	2.116
0.01499	0.01002	0.0800	0.1028	0.0349	2.579	2.107	2.119
0.00999	0.00498	0.0900	0.1036	0.02755	2.454	2.113	2.119
0.00747	0.00506	0.0900	0.1018	0.0466	2.775	2.106	2.120
0.03002	0.01505	0.1700	0.2080	0.00715	2.102	2.061	2.062
0.02747	0.01505	0.1700	0.2063	0.01345	2.209	2.076	2.063
0.01998	0.01000	0.1800	0.2060	0.0145	2.226	2.072	2.063
0.01499	0.01000	0.1800	0.2030	0.0339	2.556	2.065	2.064
0.00999	0.00498	0.1900	0.2037	0.0274	2.444	2.079	2.064
0.00747	0.00502	0.1900	0.2019	0.0456	2.753	2.073	2.064
0.03002	0.01504	0.2700	0.3082	0.00655	2.093	2.034	2.038
0.02744	0.01500	0.2700	0.3064	0.0128	2.198	2.052	2.038
0.02498	0.01501	0.2700	0.3051	0.01915	2.306	2.033	2.038
0.01999	0.01003	0.2800	0.3061	0.0140	2.218	2.041	2.039
0.01748	0.01003	0.2800	0.3045	0.0223	2.358	2.033	2.038
0.01499	0.01003	0.2800	0.3031	0.0332	2.543	2.027	2.038
0.01200	0.00998	0.2800	0.3018	0.05295	2.879	2.031	2.038
0.00999	0.00504	0.2900	0.3037	0.0269	2.436	2.011	2.039
0.00747	0.00508	0.2900	0.3019	0.04555	2.752	2.041	2.038

As a result of the measurements we obtain the following formulae for the two dissociation constants of dihydroxytartaric acid at 25° C (the square-root terms have been calculated according to Debye and Hückel)

$$-\log K_1 = 1.947 - 1.013 \sqrt{I} + 0.74\ I \quad (3)$$

$$-\log K_2 = 4.004 - 2.026 \sqrt{I} + 1.64\ I \quad (4)$$

Values calculated by means of these formulae are, for comparison, given in the fourth and sixth column of Table 2. If we extrapolate to zero ionic strength by means of the formulae, we find the activity constants $K_1 = 1.13 \times 10^{-2}$

Table 4. The first dissociation constant of tartronic acid at 37° C.

a	*b*	*s*	*I*	*E*	$-\log h$	$-\log K_1$	$-\log K_1$ (calc.)
0.02995	0.01500	0.0700	0.1076	0.00805	2.120	2.114	2.124
0.01995	0.00999	0.0800	0.1058	0.0155	2.243	2.125	2.125
0.00998	0.00500	0.0900	0.1035	0.02885	2.460	2.127	2.127
0.02995	0.01500	0.1700	0.2079	0.00745	2.104	2.067	2.069
0.01995	0.00999	0.1800	0.2060	0.01515	2.229	2.081	2.070
0.00998	0.00500	0.1900	0.2036	0.02865	2.449	2.085	2.071
0.02995	0.01500	0.2700	0.3082	0.00675	2.092	2.032	2.044
0.01995	0.00999	0.2800	0.3061	0.0146	2.220	2.052	2.045
0.00998	0.00500	0.2900	0.3037	0.02815	2.438	2.043	2.045

Table 5. The second dissociation constant of tartronic acid at 25° C.

a	b	s	I	E	$-\log h$	$-\log K_2$	$-\log K_2$ (calc.)
0.00748	0.01499	0.0700	0.1077	0.13445	4.264	4.247	4.247
0.004985	0.00999	0.0800	0.1051	0.1348	4.268	4.248	4.250
0.002492	0.004993	0.0900	0.1026	0.1357	4.282	4.254	4.252
0.00748	0.01499	0.1700	0.2077	0.1294	4.172	4.152	4.153
0.004985	0.00999	0.1800	0.2052	0.1298	4.178	4.155	4.154
0.002492	0.004993	0.1900	0.2026	0.1312	4.200	4.167	4.155
0.00748	0.01499	0.2700	0.3077	0.1261	4.117	4.096	4.116
0.004985	0.00999	0.2800	0.3052	0.1268	4.128	4.103	4.117
0.002492	0.004993	0.2900	0.3027	0.12855	4.156	4.121	4.116

and $K_2 = 9.9 \times 10^{-5}$. The only value reported in the earlier literature is $K_1 = 1.24 \times 10^{-2}$ found in the year 1898 by Skinner [5] by conductance at 25° C.

THE DISSOCIATION CONSTANTS OF TARTRONIC ACID

Tartronate buffers for the glass electrode measurements were made up from solutions prepared as follows. Ten millimoles of sodium dihydroxytartrate were added to about 100 ml water containing 5 millimoles hydrochloric acid. The mixture was heated to 80° C, and was, under gentle agitation, kept at that temperature until all the sodium salt had gone into solution under evolution of carbon dioxide (about 15 minutes). The solution was left at room temperature for one or two days to secure complete decarboxylation. The remaining carbon dioxide was removed by a current of pure air, and the solution was diluted to 200 ml in a graduated flask.

The results of the measurements of the two dissociation constants at the temperatures 25 and 37° C are shown in Tables 3—6. In the computation of K_1 from the experimental data given in Tables 3—4, a correction was made for the amount of divalent tartronate ion present by means of K_2 calculated from formulae (7) — (8). Similarly, in the computation of K_2 from the experimental data given in Tables 5—6, a correction was made for the amount of

Table 6. The second dissociation constant of tartronic acid at 37° C.

a	b	s	I	E	$-\log h$	$-\log K_2$	$-\log K_2$ (calc.)
0.00749	0.01499	0.0700	0.1077	0.1396	4.266	4.250	4.249
0.004985	0.00999	0.0800	0.1051	0.1402	4.274	4.254	4.253
0.002493	0.004996	0.0900	0.1026	0.14095	4.284	4.256	4.257
0.00749	0.01499	0.1700	0.2077	0.13425	4.169	4.150	4.144
0.004985	0.00999	0.1800	0.2052	0.13475	4.176	4.153	4.146
0.002493	0.004996	0.1900	0.2026	0.1351	4.180	4.146	4.148
0.00749	0.01499	0.2700	0.3077	0.1307	4.110	4.090	4.098
0.004985	0.00999	0.2800	0.3052	0.1313	4.119	4.094	4.099
0.002493	0.004996	0.2900	0.3027	0.3121	4.131	4.093	4.100

undissociated tartronic acid present by means of K_1 calculated from formulae (5) — (6). The results of the computation of K_1 and K_2 are shown in the next to the last column of Tables 3—6. When Debye-Hückel's limiting law, extended by a linear term, is applied, it is found that the results may best be expressed by the formulae

$$\text{(at 25° C)} \qquad -\log K_1 = 2.366 - 1.013\sqrt{I} + 0.76\, I \qquad (5)$$

$$\text{(at 37° C)} \qquad -\log K_1 = 2.380 - 1.038\sqrt{I} + 0.78\, I \qquad (6)$$

$$\text{(at 25° C)} \qquad -\log K_2 = 4.735 - 2.026\sqrt{I} + 1.64\, I \qquad (7)$$

$$\text{(at 37° C)} \qquad -\log K_2 = 4.758 - 2.076\sqrt{I} + 1.60\, I \qquad (8)$$

Values calculated by means of these formulae are given in the last column of Tables 3—6. A close inspection of the data reveals a small systematic deviation from the equations. Thus the values of $-\log K_1$ calculated from formula (5) and shown in Table 3 are on an average 0.004 too high when I is about 0.1, 0.008 too low when I is about 0.2, and 0.004 too high when I is about 0.3. The data in the three other tables show a similar departure. There may, therefore, be some doubt of the accuracy of the activity constants obtained by extrapolation to zero ionic strength by means of formulae (5) — (8), namely, $K_1 = 4.31 \times 10^{-3}$ and $K_2 = 1.84 \times 10^{-5}$ at 25° C, and $K_1 = 4.17 \times 10^{-3}$ and $K_2 = 1.75 \times 10^{-5}$ at 37° C. The only value reported in the earlier literature is $K_1 = 5 \times 10^{-3}$ found by Skinner [5] by conductance at 25° C.

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The Uncatalysed and the Cupric-Ion Catalysed Decarboxylation of Dihydroxytartaric Acid

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The decomposition of dihydroxytartaric acid into tartronic acid and carbon dioxide was studied kinetically at the temperature 37° C in solutions of hydrochloric acid and in acetate buffer solutions. By an analysis of the experimental data, separate rate constants were determined for each of the three simultaneous reactions: decarboxylation of undissociated dihydroxytartaric acid, of its univalent and divalent ion. It was found that the decomposition of the univalent ion, but not that of the divalent ion, is accelerated by acetate ions. An analogous effect of acetate ions has earlier been found in the decarboxylation of oxaloacetic acid.

It was shown that the decarboxylation of dihydroxytartaric acid, like that of other *dibasic* β-keto carboxylic acids, is catalysed by cupric ions. It was concluded from the experiments that, what appears as a cupric-ion catalysis, is actually a spontaneous decarboxylation of a complex of one cupric ion and one divalent dihydroxytartrate ion. An analogous explanation was earlier given to the cupric ion catalysis in the decarboxylation of oxaloacetic acid.

In the present paper, the decarboxylation of dihydroxytartaric acid

$$HOOC-C(OH)_2-C(OH)_2-COOH \rightarrow HOOC-CHOH-COOH + CO_2 + H_2O$$

has been studied kinetically at the temperature 37.0° C in solutions of hydrochloric acid and in acetate buffer solutions. All the solutions contained potassium chloride up to a total ionic strength of approximately 0.3. The object of the study was to make out which contribution the spontaneous decomposition of the different molecular species (undissociated acid, uni- and di-valent dihydroxytartrate ion) give to the total rate. It seems to be a general rule that the decarboxylation of dibasic β-keto carboxylic acids is catalysed by certain metal ions, *e. g.* cupric ions [1]. It was therefore of interest to examine whether cupric ions catalyse also the decarboxylation of dihydroxytartaric acid, and an attempt was made to study this question.

The reaction was followed by observing the pressure above the solution by means of an apparatus described earlier [2]. The difference (P cm mercury)

between the final pressure reading and the reading at the time t minutes after the reaction was started is proportional to the concentration of dihydroxytartaric acid present in the solution at the time t. A plot of log P against t should therefore, if the reaction is of first order, give a straight line whose slope is $k^* = 0.4343\ k$, where k denotes the rate constant. While the author in earlier papers on rates of reactions has always given k^* *per minute*, the rate constants are in this paper given as k *per second* as recommended by the Commission on Kinetics of Chemical Reactions under the International Union of Pure and Applied Chemistry [3]. Change to the new unit may be carried out by means of the equation

$$k \text{ (per second)} = 3.838 \times 10^{-2}\ k^* \text{ (per minute)}$$

MATERIALS

Sodium dihydroxytartrate was prepared and purified as described in the previous paper [4]. Owing to the slight solubility of this salt, it could only be used in kinetic experiments where the solution contained an excess of strong acid. The following two preparations of free dihydroxytartaric acid were therefore made.

Dihydroxytartaric acid (preparation 1). Through a mixture of 15 g of sodium dihydroxytartrate and 80 ml of acetone, immersed in an ice bath, was passed a current of dry hydrogen chloride. The precipitate, consisting of sodium chloride and a little unchanged sodium dihydroxytartrate, was isolated by suction on a sintered glass filter. Hydrogen chloride and acetone were removed from the filtrate by distillation in vacuum at room temperature, at last in a desiccator over sulphuric acid and solid potassium hydroxide. The brown residue was extracted with 75 ml of warm acetone. After filtration, the solution was cooled in ice and added 200 ml of benzene. The precipitate was isolated by suction, washed with benzene, and recrystallised once more by the same procedure. It was finally dried in vacuum over paraffin-wax shavings and solid potassium hydroxide.

Dihydroxytartaric acid (preparation 2) was prepared from dihydroxyfumaric acid [5] by the method of Fenton [6]. It was recrystallised in the same way as preparation 1.

Although the two preparations of the acid were kept in a desiccator over concentrated sulphuric acid, they gradually changed into tartronic acid, though with different rates. This follows from the observation that the total pressure increase in the kinetic experiments was too small when the free acid was used instead of the sodium salt, and the discrepancy increased with the age of the preparation, more rapidly for preparation 1 than for preparation 2. The same result is obtained from the following titrations. Samples of preparations 1 and 2 were taken at different time, weighed and dissolved in water. The solutions were left for 24 hours at 37° C, after which time the dihydroxytartaric acid was completely transformed into tartronic acid and carbon dioxide. The latter was removed by a current of carbon-dioxide-free air, and the solution was titrated with sodium hydroxide. From the results the following data were obtained:

	Preparation 1					Preparation 2		
Age of the preparation (days)	9	20	28	39	64	17	36	47
Mole per cent tartronic acid	5	17	26	38	62	0	7	9

Preparation 1 was not more than 35 days old, preparation 2 not more than 50 days old, when used in the kinetic measurements.

The water, hydrochloric acid, and acetic acid used for making up the solutions were redistilled in an all-glass apparatus. The potassium chloride was of analytical purity and was further recrystallised twice from redistilled water. The acetate buffers were prepared from stock solutions of potassium chloride, acetic acid, and potassium acetate. The latter was made by mixing titrated solutions of acetic acid and potassium hydroxide. When a

solution of potassium chloride prepared by neutralising potassium hydroxide with hydrochloric acid, instead of one made from crystalline potassium chloride, was used for making up the acetate buffer, the same rate constant was obtained. This shows that the potassium hydroxide did not contain perceptible amounts of catalytically active impurities.

THE RATE OF DECOMPOSITION IN SOLUTIONS OF HYDROGEN AND POTASSIUM CHLORIDE

The composition of the solutions examined and the results obtained are shown in Table 1. In the first column, the word Salt indicates that the solution of dihydroxytartaric acid was made, and the reaction started, by dissolving the sodium salt in a solvent containing an excess of hydrochloric acid, while Ac. 1 and Ac. 2 denote that preparation 1 and 2, respectively, of the free acid were dissolved. The initial concentrations x_0 of dihydroxytartaric acid are given in the third column. The ionic strengths of the solutions varied only from 0.301 to 0.307.

Table 1. Decomposition of dihydroxytartaric acid at 37° C.

Prep.	Curve Fig. 1	x_0	HCl	KCl	NaCl	α_1	α_2	$k\ 10^4$ found	$k\ 10^4$ calc.
Salt		0.01374	0.2720		0.027	0.0821	0.00013	0.381	0.380—0.381
Ac. 1		0.01551	0.1997	0.100		0.1083	0.00023	0.489	0.489—0.491
Salt		0.01376	0.1721	0.100	0.028	0.1234	0.00030	0.552	0.552—0.555
Salt		0.01379	0.0722	0.200	0.028	0.2467	0.00138	1.082	1.071—1.087
Ac. 2	II	0.01377	0.0500	0.250		0.3151	0.0025	1.368	1.354—1.405
Salt		0.01373	0.0426	0.230	0.027	0.3450	0.0031	1.517	1.487—1.540
Salt		0.01320	0.0235	0.250	0.026	0.461	0.0069	2.062	1.999—2.113
Salt	I	0.01392	0.0220	0.250	0.028	0.471	0.0073	2.089	2.038—2.169
Ac. 1		0.01367	0.0100	0.290		0.587	0.0149	2.72	2.59 —2.83
Ac. 2		0.01372	0.0100	0.290		0.587	0.0149	2.72	2.59 —2.83
Ac. 2	III	0.01380		0.300		0.708	0.0342	3.67	3.35 —3.75
Ac. 1	IV	0.01389		0.300		0.708	0.0342	3.65	3.35 —3.75

If the hydrogen ion concentration is constant, we may expect a reaction of first order with the constant

$$k = k_0\ (1—\alpha_1—\alpha_2) + k_1\ \alpha_1 + k_2\ \alpha_2 \qquad (1)$$

where k_0, k_1, and k_2 are rate constants for the spontaneous decomposition of undissociated dihydroxytartaric acid, univalent and divalent ion, respectively, while α_1 and α_2 denote the degrees of dissociation into uni- and di-valent ion, respectively. Since tartronic acid is weaker than dihydroxytartaric acid, the hydrogen ion concentration will actually decrease during the reaction. A departure from the first-order law may therefore be expected, especially when little or no hydrochloric acid is present. No significant deviation from the first-order law could, however, be detected in those experiments where the solution was made by dissolving the sodium salt. In Fig. 1, Curve 1, is shown

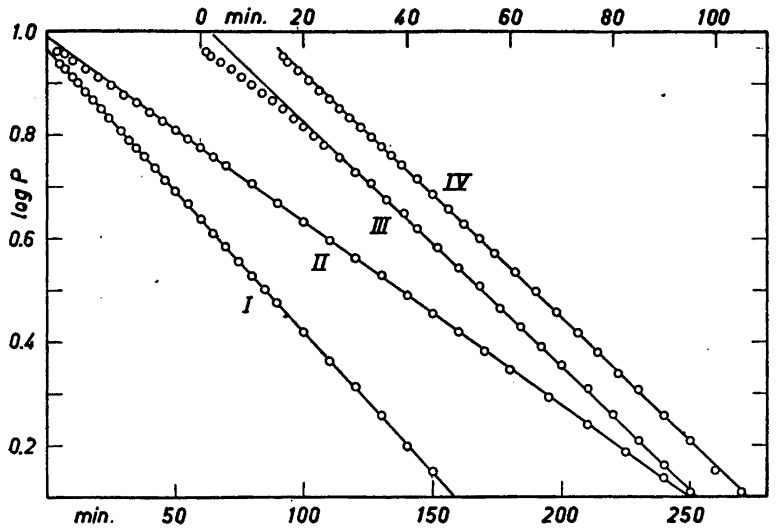

Fig. 1. Plots of log P *against* t *for four of the experiments presented in Table 1. The time scale for Curves I and II is given at the bottom, that for Curves III and IV at the top. Curve IV has been displaced a length corresponding to 15 minutes to the right.*

a plot of log P against t for that solution where the largest deviation should be expected. When the free acid was dissolved instead of the sodium salt, a systematic deviation, larger for preparation 2 than for preparation 1, was found in the initial stages of the reaction as shown in Fig. 1, Curves II—IV. This deviation could, however, not be explained by the change in α_1 and α_2 due to the formation of tartronic acid. Except in the first part of the reaction, the points fell, also here, close to straight lines, from which the rate constants k given in the next to the last column of Table 1 were computed.

The degrees of dissociation α_1 and α_2 of dihydroxytartaric acid, both those corresponding to the initial and to the final composition of the solutions, were computed by means of the dissociation constants of dihydroxytartaric acid and tartronic acid found in the previous paper [4]. Since the dissociation constants of dihydroxytartaric acid were not measured at 37° C, those corresponding to 25° C were applied. The values used were: for dihydroxytartaric acid $K_1 = 2.44 \times 10^{-2}$ and $K_2 = 4.2 \times 10^{-4}$, for tartronic acid $K_1 = 9.02 \times 10^{-3}$ and $K_2 = 8.1 \times 10^{-5}$. The degrees of dissociation presented in Table 1 are averages of those calculated for the initial and final composition of the solutions. Equation 1 was then applied to the experimental data. For k_2 was chosen the value 17.7×10^{-4} obtained from experiments on acetate buffers. The best agreement with the measurements was found when

$$k_0 = 0.041 \times 10^{-4} \text{ sec}^{-1} \quad \text{and} \quad k_1 = 4.15 \times 10^{-4} \text{ sec}^{-1}$$

By means of eqn. (1) and the data for k_0, k_1, and k_2 given here, we may calculate k when α_1 and α_2 are known. In the last column of Table 1 are given two values for each solution, the former has been calculated for the initial, the latter

for the final composition of the solution. The value of k obtained directly from the measurements is always within the interval determined by the two calculated values. When the last two experiments are excepted, it is always very close to their average.

THE RATE OF DECOMPOSITION IN ACETATE BUFFER SOLUTIONS

The reaction was started by dissolving free dihydroxytartaric acid in solutions containing acetic acid, potassium acetate, and potassium chloride. The experiments presented in Table 2 were all carried out with preparation 1 of the acid. They agreed well with the first-order law. A plot of $\log P$ against t for the solution having the smallest buffer capacity (the last one in Table 2) is shown in Fig. 2, Curve V. When preparation 2 was used instead of preparation 1, a rather large deviation from first-order was observed in the first part of the reaction (similar to that shown in Fig. 1, Curves II—IV), but the points approached a straight line having the same slope as that found for preparation 1. No explanation can be given to the different initial rates of the two preparations.

Table 2. Decomposition of dihydroxytartaric acid (initial concentration 0.0146 molar) at 37° C in the solvent: a *molar acetic acid,* b *molar potassium acetate, (0.300 —* b*) molar potassium chloride.*

a	b	[HAc]	[Ac^-]	α_1	k 10^4 found	k 10^4 calc.	k_1 10^4	k_2 10^4
0.400	0.300	0.426	0.274	0.106	17.28	17.31	14.5	17.64
0.200	0.300	0.227	0.273	0.059	17.48	17.46		
0.100	0.300	0.128	0.272	0.034	17.56	17.53		
0.040	0.300	0.068	0.272	0.019	17.55	17.58		
0.400	0.200	0.424	0.176	0.155	16.79	16.77	12.4	17.58
0.200	0.200	0.226	0.174	0.090	17.09	17.11		
0.100	0.200	0.127	0.173	0.053	17.29	17.30		
0.040	0.200	0.068	0.172	0.029	17.44	17.43		
0.300	0.100	0.323	0.077	0.242	15.39	15.39	7.7	17.83
0.200	0.100	0.224	0.076	0.183	15.97	15.98		
0.100	0.100	0.125	0.075	0.113	16.77	16.69		
0.040 [a]	0.100	0.067	0.073	0.065	17.09	17.17		

[a] The kinetic data for this solution are shown in Fig. 2, Curve V.

In order to compute k_1 and k_2 from the experimental data, we apply eqn. (1). When the small amount of undissociated dihydroxytartaric acid present in the solution is neglected, the equation may be written

$$k = k_2 - (k_2 - k_1)\ \alpha_1 \qquad (2)$$

The hydrogen ion concentration of the solution decreases slightly when dihydroxytartaric acid is transformed into tartronic acid. α_1 was therefore computed both for the initial and the final composition of the solution. The values of α_1 given in Table 2 are averages of the two values. The concentrations of undissociated acetic acid [HAc] and the acetate ion concentration [Ac^-] given in the third and fourth column of the table are also those corresponding to an average composition of the solution. In the computation of α_1, the dissociation constants of dihydroxytartaric and tartronic acid given earlier in this paper were used, while for acetic acid was chosen $K = 3.20 \times 10^{-5}$ (estimated [7] for 0.3 molar sodium chloride as solvent from measurements by Harned and Hickey [8]). Owing to inaccuracies in the dissociation constants applied, the values of α_1 given in the table may all be either a little too high or a little too low. This will not perceptibly affect the value found for k_2, since this constant is determined by a short extrapolation to $\alpha_1 = 0$ by means of eqn. (2). It may, however, have an appreciable influence in the computation of k_1, but the determination of k_1 from experiments on acetate buffer solutions is already less accurate since here α_1 is always much smaller than α_2.

Within each of the three series of solutions presented in Table 2, the acetate ion concentration is nearly constant. Applying eqn. (2) to each series separately, we compute the values of k_1 and k_2 given in the last two columns of the table. When they are introduced into eqn. (2), and k is calculated, the values given in the seventh column are obtained. When the three series of measurements are compared, it is seen that k_2 is independent of the acetate ion concentration. The average value for all three series is $k_2 = 17.7 \times 10^{-4}$ sec^{-1}. The values of k_1 increase, however, with increasing acetate ion concentration as shown in the following lines

[Ac^-]	0	0.075	0.174	0.273
k_1 10^4	4.15	7.7	12.4	14.5
$\Delta k_1 10^4/[Ac^-]$		47	47	38

An analogous effect was found in the decarboxylation of oxaloacetic acid [7]. Also here, the rate constant for the decomposition of the divalent ion was independent of the acetate ion concentration, while that for the decomposition of the univalent ion increased with increasing acetate ion concentration, although relatively less than in the case of dihydroxytartaric acid.

The rate constants for the spontaneous decarboxylation of four β-keto carboxylic acids and their ions at 37° C are, for comparison, given in Table 3. Their dissociation constants are shown in the last two columns of the table.

CUPRIC-ION CATALYSIS

The solutions examined contained hydrogen chloride, potassium chloride, and cupric nitrate, and the reaction was started by adding sodium dihydroxytartrate. It was found that cupric ions have a large catalytic effect, which increases rapidly with decreasing hydrogen ion concentration. The kinetics of the reaction was, however, except in the most acid solutions examined, too

Table 3. Rate of decarboxylation of some β-keto carboxylic acids. k_0, k_1, *and* k_2 *are the rate constants in* sec^{-1} *for the spontaneous decarboxylation of the undissociated acid, the univalent ion, and the divalent ion, respectively, at the temperature 37° C.* K_1 *and* K_2 *are the first and second dissociation constant of the acid at 25° C and zero ionic strength.*

	$k_0\ 10^4$	$k_1\ 10^4$	$k_2\ 10^4$	$K_1\ 10^3$	$K_2\ 10^4$
Dihydroxytartaric acid [a]	0.041	4.15	17.7	11.3	0.99
Oxaloacetic acid [a]	0.068	2.48	0.695	2.79	0.427
Acetoacetic acid [b]	0.80	0.0148		0.27	
Dimethylacetoacetic acid [c]	3.15	0.0091		0.31	

[a] The rate constants are for the solvent 0.3 M KCl. Pedersen [4,7,9] and this paper.
[b] Ljunggren [10], Pedersen [11].
[c] The rate constants are extrapolated to 37° from data at 25° and 35° C. Pedersen [12].

complicated to make an analysis of the data seem promising. An attempt to give a partial interpretation of the kinetic data was therefore only made for the two series of measurements presented in Table 4. When log P in these experiments was plotted against t, it was only within a limited range that the points did not deviate systematically from a straight line. Before this interval the points were below, after it they were above the straight line. This is shown in Fig. 2, Curves VI and VII, for two of the solutions in Table 4. In the fourth column of the table is shown how many per cent of the dihydroxytartaric acid

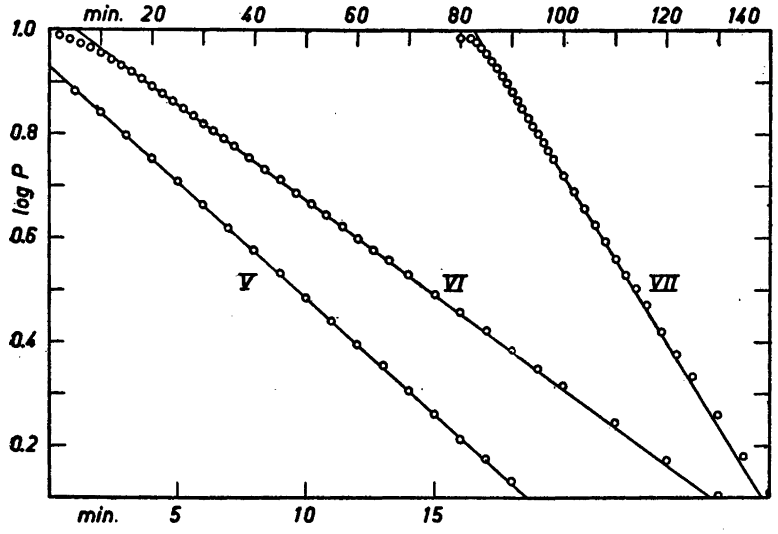

Fig. 2. Plots of log P *against* t *for one experiment in Table 2 (Curve V) and for two experiments in Table 4 (Curves VI and VII). The time scale for Curve V is given at the bottom, that for Curves VI and VII at the top. Curve VII has been displaced a length corresponding to 80 minutes to the right.*

Table 4. Cupric ion catalysis in the decarboxylation of dihydroxytartaric acid (initial concentration 0.014 M) at 37° C.

Solvents. A: 0.2720 M HCl, 0.028 M NaCl, c molar $Cu(NO_3)_2$
B: 0.1721 M HCl, 0.100 M KCl, 0.028 M NaCl, c molar $Cu(NO_3)_2$

Solvent	c	Curve in Fig. 2	Range %	k 10^4	k_c 10^4	$\frac{k_c}{c}$ 10^2	a_2 10^4	$\frac{k_c}{ca_2}$
A	0.00000			0.381	0.000		1.27	
A	0.00100		6—60	0.855	0.474	4.74		373
A	0.00200		6—70	1.328	0.947	4.74		
A	0.00500	VI	20—80	2.79	2.41	4.82		
A	0.01000		37—83	5.33	4.95	4.95		
A	0.1000		30—95	46.9	46.5	4.65		
B	0.00000			0.552	0.00		2.99	
B	0.00200		10—43	2.79	2.24	11.2		375
B	0.00500	VII	20—65	6.19	5.64	11.3		
B	0.01000		70—95	11.0	10.4	10.4		

was decomposed at the beginning and at the end of the range within which the first-order law was obeyed. The rate constants k given in the fifth column were computed in the usual way from the slopes of the straight lines. When the value of k found for the solvent without cupric nitrate was subtracted, the rate constant k_c for the pure cupric ion catalysis was obtained. It is seen from the table that k_c is, in both series, nearly proportional to the cupric ion concentration c, but the proportionality factor k_c/c increases with decreasing hydrogen ion concentration. As shown in the last column of the table, k_c/ca_2 has, however, the same value in both series. It is therefore reasonable to conclude that, what appears as a cupric-ion catalysis, is actually a spontaneous decarboxylation of a complex CuT of one cupric ion and one divalent dihydroxytartrate ion, T^{--}. When x denotes the analytical concentration of dihydroxytartaric acid, k_{CuT} the rate constant for the spontaneous decarboxylation of the complex CuT, and K_{CuT} the complexity constant, we have

$$k_c\, x = k_{CuT}\, [CuT] = k_{CuT}\, K_{CuT}\, c\, a_2\, x$$

Hence,

$$k_c/ca_2 = k_{CuT}\, K_{CuT}$$

It therefore follows from the data in Table 4 that the product of the rate constant for the spontaneous decarboxylation of the cupric-dihydroxytartrate complex and its complexity constant is about 374 $sec^{-1}mole^{-1}$ l at the temperature 37° C and the ionic strength 0.3.

An analogous explanation was in an earlier paper [1] given to the cupric-ion catalysis in the decarboxylation of oxaloacetic acid. The catalysis was here explained as being the result of a spontaneous decarboxylation of a complex CuOx of one cupric ion and one oxaloacetate ion, Ox^{--}. The reaction was studied at 37° C and at ionic strengths up to 0.206. The values found for the product of the rate constant k_{CuOx} for the spontaneous decarboxylation of the

complex CuOx and its complexity constant K_{CuOx} were, when recalculated to the units used in the present paper:

Ionic strength	0.053	0.104	0.206	(0.3)
$k_{CuOx}K_{CuOx}$($sec^{-1}mole^{-1}l$)	1 080	690	421	(360)

The value of the product corresponding to the ionic strength 0.3 was found by extrapolation. It is interesting to note that the product of the rate constant and the complexity constant is nearly the same for the cupric-dihydroxytartrate and the cupric-oxaloacetate complex. For the latter it was possible to give approximate values of the two factors. It was found that the rate constant for the decarboxylation of the cupric-oxaloacetate complex k_{CuOx} is about 5.8×10^{-2} sec^{-1} independently of the ionic strength. If the complexity constants for the two complexes are of the same order of magnitude, the rate constants for their decarboxylation will also be of the same order of magnitude.

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Polyacids from Polyacrylonitrile. Viscosimetry in Pure Aqueous Solution

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The viscometric behaviour of polyelectrolytes, obtained by alkaline saponification of polyacrylonitrile, has been compared with that of polyacrylic acid, prepared directly from the monomer by redox polymerization in aqueous solution. It is apparent that differences exist, but their qualitative significance may be interpreted in terms of the Flory theory on general frictional properties of macromolecules.

When a suspension of polyacrylonitrile is heated to 75—80° C in strong (10—30 %) sodium hydroxide solution, a saponification of the nitrile groups takes place, finally resulting in a viscous solution of sodium polyacrylate. The base should be present in a great excess. The polymer formed shows a limiting nitrogen content of 1.5—2.0 % after purification, but solubility in water occurs much earlier than that value has been reached. A complete removal of the nitrile groups has not been successful in the present investigation, and it is doubtful if this can be carried out in the present way. From analytical specifications [1] it is known that only 94—95 % of the nitrile groups in the polymer are accessible to alkaline saponification in aqueous media.

Two reaction steps before dissolution can be identified by the appearance of distinct colours. The first one is a change to "*carotene*" colour, accompanied by a low degree of swelling. Simultaneously the polymer loses its property of beeing soluble in dimethyl formamide. As the reaction proceeds, a gel state is obtained and the colour changes into that of *lycopene*. Most of the ammonia gas is liberated during this step. When the gel gradually dissolves, the colour turns into a faint yellow one.

The purification and isolation of the polyelectrolyte is comparatively intricate. The large excess of sodium hydroxide, indispensable for a proper handling of the reacting polymer, makes precipitation with sulfuric acid rather inattractive. Moreover, this method has to be combined with a dialysis, preferably amplified with a reprecipitation [2]. We have noticed, however, that only a minor amount of methanol, added to the alkaline solution, is sufficient for the separation of the polymer as a white curd. A further washing with pure

methanol reduces the plasticity, thus gives a more rigid structure, but does not remove the free base. It seems questionable if the alcohol penetrates the polymer phase as syneresis takes place when the water is extracted. According to our present experience the simplest way for a further purification is adding pure water to the centrifuged specimen and decant after some hours. A moderate swelling of the polymer takes place, but only a minor fraction dissolves. The operation can be repeated carefully, until a stage is reached where swelling and dissolution is rapid. This behaviour seems to be an analogy to the increase of the thickness of the Debye-Hückel ionic atmosphere around (the) *polyions* at increasing dilution. The successive removal of sodium hydroxide by "dialysis" in water reduces the compressive forces, acting on the polyions, even in the gel state. An increase in swelling is therefore expected, but also a gradual diffusion of the *counter* ions from their original position into the regions previously occupied by pure solvent. But this will cause an increase of the net charge of the polyanions (intramolecular Coulomb repulsion), finally ending in complete dissolution. The point of view is equivalent in meaning of that presented by Fuoss and Strauss [3], valid for the reverse case, *i. e.* screening of a polyelectrolyte by addition of inorganic electrolytes. — Of course the presence of methanol in the precipitated polymer must have some influence on the rate of swelling, but not on the efficiency of the operation as a whole. If desirable the main fraction of the alcohol can be removed *in vacuo* before the final purification, but no tenable reason could be found for this more tedious operation.

Solutions of sodium polyacrylate, obtained in this way, may still show a slight excess of base, but a final purification can be carried out by adding a controlled amount of hydrogen-saturated cation exchanger to the aqueous system as a finely divided suspension. An excess of ion exchanger reduces the pH value rapidly, reaching the limiting value of 5.1 within a few minutes. After filtering or decanting the solution can be used directly for viscometry.

The viscometric behaviour of polyelectrolytes is extensively discussed in the fundamental papers of Fuoss and Strauss [3,4], and Kern [5]. A study on polyacrylic acid, prepared by reduction activation of acrylic acid in aqueous solution, has been carried out by Markowitz and Kimball [2]. In most cases a rapid increase of the numerical value of the viscosity number (η_{sp}/c) has been observed at increasing dilution, provided no inorganic electrolytes are present. Recent investigations [5,6,10], however, indicate deviations from this rule at exceedingly dilute systems, and our present study on saponified polyacrylonitrile seems to confirm these observations. Obvious difficulties in measuring viscosities at very high dilutions still makes it uncertain to draw any conclusion of general validity, but it seems clear that not all experimental data can be handled satisfactorily according to the empirical Fuoss-Strauss equation [4]

$$\eta_{sp}/c = A/(1 + B \cdot c^{1/2}),$$

where η_{sp}/c is the viscosity number, c concentration of the polyion in monomer units per unit of volume, A and B constants. In other formulation, a plot of $(\eta_{sp}/c)^{-1}$ against $c^{1/2}$ would indicate a straight line.

The present study was carried out in order to compare the viscometric behaviour of the polyacids, obtained by saponification of polyacrylonitrile, with the corresponding polymer from pure polyacrylic acid. The main difference in structure is believed to be the presence of unsaponified nitrile groups in the former compound; a linear structure is thus tacitly assumed in both chain molecules. On account of this the first type should be considered as a random copolymer of acrylic acid and acrylonitrile. The last mer unit decreases the solubility in water (the entrapment of solvent in the Huggins [8] theory), why a change of the numerical value of the expansion factor α in the Flory [9] treatment of the frictional properties of polyelectrolytes should be expected.

Special attention was devoted to the preparation of the polymers in order to maintain most similar polymerization conditions and a high degree of purity (see experimental part). Here should be mentioned only the principal details. The acrylonitrile was a commercial grade, prepared from ethylene oxide and substantially free from bifunctional impurities. The acrylic acid was obtained from the same monomer by hydration with strong sulfuric acid in the presence of copper powder and hydroquinone, followed by distillation *in vacuo* over copper wire and repeated crystallization of the main fraction. Polymerization was initiated in homogenous system (aqueous solution) by identical activators in all cases. The polyacrylonitrile was reprecipitated from ethylene carbonate by dilution with water. The polymer from acrylic acid was neutralized with sodium hydroxide, precipitated in methanol and purified with ion exchanger as described above.

EXPERIMENTAL

Preparation of polyacrylonitrile and saponification to sodium polyacrylate (random copolymer). The freshly distilled monomer was mixed with water at 50° C (40 g acrylonitrile in 1 000 ml water) in a flask with three necks, equipped with agitator, thermometer, reflux condensor and inlet for nitrogen gas. On slow agitation the activator system was added as separate solutions of 0.20 g ammonia persulphate and 0.30 g sodium pyrosulphite in small amounts of water. The initiation of polymerization was very rapid and within five minutes a thick slurry of finely divided polymer was obtained. It was collected on a Pyrex G3 glass filter, washed with distilled water at 50° C, with acetone at 20° C and with tepid distilled water again. The wet cake was used directly for saponification after determination of dry content at 110° C on a small sample. The specimen was dispersed in water and a filtered aqueous solution of sodium hydroxide (45 % by weight) was added to give a reaction mixture, approximately containing 5 % polymer in 20 % sodium hydroxide solution. This dispersion was heated on a water bath to 70° C for several hours (62 hours for polymer I, 40 hours for polymer II, and 36 hours for polymer III). After cooling to room temperature, methanol (250 ml, distilled over sodium hydroxide flakes) was added to precipitate the polymer. The soft putty, immediately obtained, was soaked in pure methanol, repeatedly in distilled water until a rapid swelling was observed, diluted to a large volume (solid content about 1 %) and purified with ion exchanger (Amberlite IR-120, hydrogen saturated) as mentioned above.

Preparation of sodium polyacrylate from acrylic acid. Crystals of glacial acrylic acid were dissolved in distilled water to give a 4 % solution (40 g monomer in 1 000 ml water) and heated to 50° C before the addition of the activator system, beeing identical with that previously applied for acrylonitrile. The reaction showed a long period of induction and the expected increase in viscosity, originating from polymer formation, did not appear before at least 20 minutes had elapsed. After three hours a viscous dope had been formed. Viscosity still increased on neutralization with sodium hydroxide, but rapidly drops when the base finally is in excess. Also in this case the polymer was precipitated with methanol, soaked in water, diluted and purified with cation exchanger. The procedure was repeated to remove a possible residue of monomer (sodium acrylate).

Table 1a. Polymer I (sodium polyacrylate from polyacrylonitrile). Nitrogen content of polymer, dried at 110° C: 1.91 %. Measurements of viscosity number (η_{sp}/c) of aqueous solutions at pH 7.0. Measuring temperature 20.02°. Viscometer: Type Ubbelohde, calibration constant K = 0.010. Viscosity of water = 102.1 sec = 1.021 cst. (uncorrected value).

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
225	1 500.6	61
113	991.3	77
56.3	670.6	100
28.2	446.5	119
14.1	282.9	125
7.05	192.5	126
3.53	142.0	111
1.77	120.4	102
0.89	111.4	102

Table 1b. Polymer I (sodium polyacrylate from polyacrylonitrile), deionised with an excess of hydrogen saturated cation exchanger (Amberlite IR-120). Initial pH of solution: 5.1. Measurements of viscosity number at 20.02° C in Ubbelohde viscometer.

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
500	1 204.4	21.5
250	868.1	29.9
125	639.1	42.0
62.5	469.7	57.5
31.3	333.8	72.5
15.6	231.5	81.0
7.8	167.7	82.1
3.9	130.5	70.7
2.0	117.3	75.3

Table 2. Polymer II. Nitrogen content 2.16 %. Measurements of viscosity number at pH 7.0, temperature 20.02° C, in Ubbelohde viscometer.

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
375.0	1 362.5	32.9
187.5	905.0	41.8
93.75	642.3	56.3
46.88	423.0	66.9
23.44	228.0	52.3
11.72	178.2	63.7
5.86	140.4	64.0
2.93	120.8	62.5
1.47	111.6	63.2
0.74	106.2	56.2

Table 3a. Polymer III. Nitrogen content 2.09 %. Measurements of viscosity number at pH 7.0, temperature 20.02° C, in Ubbelohde viscometer.

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
605.0	3 077.7	48.1
302.5	1 979.8	60.7
151.3	1 245.2	74.0
75.6	852.3	97.1
37.8	523.0	108.8
18.9	300.0	102.3
9.5	197.0	98.0
4.7	137.8	73.6
2.4	119.4	71.1
1.2	110.4	67.8

Table 3b. Polymer III, deionised with an excess of hydrogen saturated cation exchanger (Amberlite IR-120). Initial pH of solution: 5.1. Measurements of viscosity number at 20.02° C in Ubbelohde viscometer.

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
605.0	666.0	9.1
302.5	494.4	12.6
151.3	370.2	17.2
75.6	281.2	23.0
37.8	214.1	28.7
18.9	167.7	33.1
9.5	135.3	33.9
4.7	117.8	32.0
2.4	108.8	27.1
1.2	105.4	26.3

Table 4a. Polymer IV (sodium polyacrylate, prepared by redox polymerization of acrylic acid). Measurements of reduced viscosity number at pH 7.0, temperature 20.02° C, in Ubbelohde viscometer.

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
312.4	3 850.5	117.3
156.2	2 099.6	125.2
78.1	1 073.5	121.8
39.0	534.4	108.4
19.5	282.5	90.4
9.8	171.0	69.0
4.9	134.5	64.8
2.5	119.2	68.2
1.2	110.7	68.2

Table 4b. Polymer IV, deionised with an excess of cation exchanger (Amberlite IR-120). Measurements of viscosity number at 20.02° C in Ubbelohde viscometer. Initial pH of solution: 5.1.

Solid content (c) (g/100 ml) × 10^3	Viscosity, sec.	Red. viscosity, η_{sp}/c
625.0	1 888.4	27.9
312.5	1 282.1	36.9
156.3	902.3	50.1
78.1	636.9	66.9
39.1	424.0	81.0
19.5	286.0	92.0
9.8	209.3	107.4
4.9	154.7	105.2
2.5	128.4	106.6
1.2	115.5	109.1

SOME COMMENTS

The change in solid content, taking place on adding ion exchanger to solutions of sodium polyacrylate has been left unconsidered, as the number of macromolecules in solution is unchanged. The ratio of the two units sodium acrylate and acrylic acid (1.3) might be included in the expressions for viscosity number, but the physical significance of this calculation is doubtful, as the polymers studied do not show identical building sequences. The general feature of the viscosity changes on dilution can, anyhow, be deduced from the semi-logarithmic diagrams in Figs. 1 and 2. Distinct maxima are apparent on all curves, representing highly ionised polymers (sodium polyacrylates Ia, II,

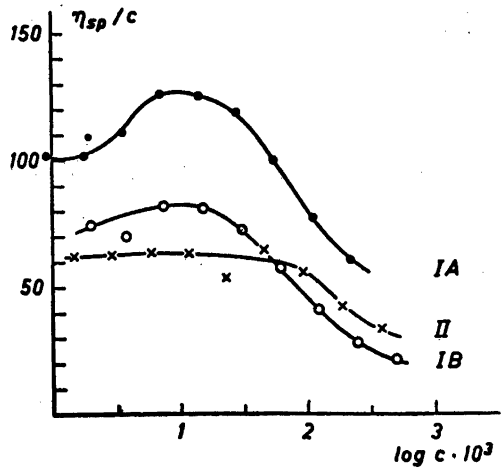

Fig. 1. Reduced viscosity of two polymers (Tab. 1 and 2), obtained by alkaline saponification of polyacrylonitrile.

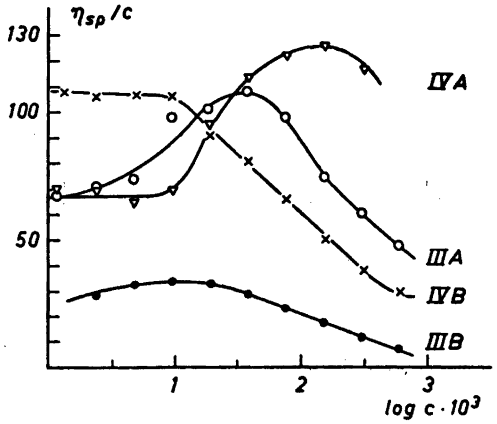

Fig. 2. Reduced viscosity behaviour of a polymer (Tab. 3), obtained by alkaline saponification of polyacrylonitrile, compared with that of a polymer, directly obtained from acrylic acid (Tab. 4).

IIIa and IVa). An asympthotic approach to the vertical axis has not been detected for any polymer, a circumstance that clearly points out the non-validity of the Fuoss-Strauss relation in the present case. The polyacids (Ib, IIIb and IVb) show maximum viscosity numbers at an average concentration of 10^{-3} g/100 ml. The straight acrylic acid polymer (IVb) keeps its (maximum) viscosity number unchanged at further dilution, but the polyacids, containing hydrophobic mer units (acrylonitrile), show a gradually decreasing value of the reduced viscosity. One is inclined to interpret this screening effect of the molecules as a return to a more coiled shape, possible when the overlap forces between separate polyions are gradually reduced. *Along* the separate chains the Coulomb field, arising from charged side groups is of course perturbated by the presence of the second mer unit in a random fashion, with the possible consequence that coiling takes place more readily than in the case of pure polyacrylic acid. This is explicitely formulated by saying that the volume expansion factors are not identical, or, that the two types of polyacids show different *Flory* temperatures.

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On the Catalytic Effect of Blood Serum on the Reaction between Colloidal Sulfur and Cyanide

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The catalytic effect of blood serum on the reaction between colloidal sulfur and cyanide has been investigated. The activity is confined to the albumin fraction, whereas the serum globulins are inactive. Low molecular weight sulfhydryl compounds are also active, although much less than the serum albumin when calculated on a molar basis. The reaction mechanism is discussed.

It was reported by Lang [1] that liver tissue catalyzed the formation of thiocyanate from colloidal sulfur and cyanide. The catalytic effect of the tissue was attributed to its content of rhodanese, an enzyme catalyzing the formation of thiocyanate from cyanide and thiosulfate. Investigations with purified rhodanese preparations showed [2], however, that this enzyme was without any effect on the reaction between colloidal sulfur and cyanide, but it was confirmed that different tissues contained a heat labile factor catalyzing this reaction. This factor, for which the name rhodanese S was suggested, was present also in blood serum in contrast to rhodanese. In this paper investigations on rhodanese S from blood serum will be reported.

METHODS

The test system described in the previous publication was abandoned, as no reproducible results were obtained due to the instability of the sulfur sol used. A new test system was developed in which a more stable sulfur sol was obtained by diluting a supersaturated solution of sulfur in ethylene glycol monoethylether with water. The assay was carried out as follows:

0.25 ml ethylene glycol monoethylether, containing 1.6 mg S per ml was added to 1.75 ml water in a small centrifuge tube, whereupon 0.5 ml sample solution, containing 0.10 M KCN and 0.04 M HCl was added. The pH obtained was 9.5. After 5 minutes at 20° C the reaction was stopped by the addition of 1 ml ferric nitrate reagent [2] and the mixture allowed to stand for 5 minutes in order to coagulate the sulfur. After the addition of 6.5 ml water and a subsequent centrifugation, the thiocyanate formed was obtained from the optical density determined at 460 mμ. In the case of some serum fractions which stabilized the sulfur sol against the effect of ferric nitrate, the optical density was corrected for remaining turbidity by adding to the contents in the cuvette 0.02 ml of a 30 %

mercuric nitrate solution. This caused the red thiocyanate color to disappear immediately but left the turbidity unaffected. A blank determination (the sample omitted) was always carried out and the corresponding correction was made. The test was calibrated with rabbit serum and the amount of CNS^- formed in the system was up to 2 μ-equivalents directly proportional to the amount of serum in the test. The activities of the different compounds are given as μ-equivalents of CNS^- formed per mg compound under the experimental conditions.

RESULTS

Using rabbit serum as a source of the factor it was observed in preliminary experiments that the pH-optimum for the catalyzed reaction was about pH 9.5, whereas the rate of the spontaneous reaction increased continuously with pH. About 50 % of the activity remained after heating the serum for 5 minutes at 60° C and only 15 % remained after 5 minutes at 70° C. Serum could be dialyzed against buffer or distilled water without significant loss of activity, and evidence was thus obtained for the protein nature of the factor. Determinations were consequently carried out on human serum (from retroplacentar blood) and protein fractions obtained thereof through a modified procedure according to Cohn *et al* [3]. From the results, shown in Table 1, it is evident that serum albumin is very active, whereas other fractions are less active or completely inactive. The activity found in these other fractions seemed to be proportional to their contamination with serum albumin, which is thus alone responsible for the serum activity, whereas the globulins are inactive. In the case of fractions IV-4 and VI the observed activities were lower than those calculated from the albumin content. In the case of fraction IV-4 it could be demonstrated that this fraction had an inhibiting effect on the serum albumin, but no such effect was obtained with fraction VI. This fraction, however, was obtained by freeze-drying the final supernatant remaining after precipitating out the other protein fractions. As this supernatant represents a very dilute (0.05 %) solution of the serum albumin it is possible that the albumin in fraction VI was to a large part denatured during the freeze-drying process. The activity and amount of protein found in fraction VI was also only about

Table 1. Catalytic activity of human serum fractions.

The albumin content was calculated from the activity by dividing the activity for each fraction by that of fraction V, assuming that the latter consisted of pure albumin.

Fraction	Activity μ-equiv.CNS^-/mg	[Albumin content] From electrophoresis *, %	[Albumin content] Calculated from the activity, %
Serum	0.60	43	42
II	0.045	5	3
III	0	Traces	0
IV–1	0.096	8–10	7.2
IV–4	0.18	30	13
V	1.40	100	100
VI	0.39	75	28

* Personal communication from Ingeniör H. Björling of AB Kabi.

Table 2. Heat stability of serum albumin.

Bovine serum albumin (Armour Fraction V) in 4 % solution of pH 7.7 heated at indicated temperature for 5 min. prior to assay.

Temperature, C°	Remaining activity, %
Control	100
40	101
45	96
50	95
55	72
60	44
70 *	12

* A slight turbidity appeared in this sample after the heat treatment whereas other samples were perfectly clear.

a few per cent of their part in the original serum. The catalytic activity of the albumin was also verified on Fraction IV from bovine serum and on crystalline bovine serum albumin, which had activities of 1.08 and 1.01 μ-equivalents CNS^{-1}/mg, respectively. That the activity was confined to the native protein and destroyed by denaturation was demonstrated by the effect of heat treatment and pH on the activity of bovine albumin (Table 2 and 3). Similarly tryptic digestion of the albumin for 14 hours at 37° C (2.5 mg trypsin and 19 mg albumin per ml at pH 9.0) destroyed 73 % of the activity. Furthermore, the activity was entirely destroyed by dodecyl sulfate at 0.001 M concentration.

Some other proteins, egg albumin, β-lactoglobulin and myoglobin, were also assayed and found to be completely inactive. Investigations on some amino acids and related compounds (Table 4) demonstrated, however, that compounds containing a sulfhydryl or a disulfide group had a certain activity, although calculated on a molar basis much less than that of serum albumin. Under the conditions of the test, cysteine thus had an activity of 1.21 equivalents CNS^-/mole which is only 1.3 % of the corresponding figure for human

Table 3. pH-Stability of serum albumin.

Bovine serum albumin (Armour Fraction V) in 2 % solution exposed to the indicated pH for 30 min. The sample neutralized prior to assay. Activity at pH 7.8 taken as 100 %.

pH	Remaining activity, %
1.8	86
3.4	97
4.1	100
5.8	101
7.8	100
9.4	101
10.7	98
11.5	70
11.9	46

Table 4. Activity of low molecular weight compounds.

Compound	Specific activity μ-equiv.CNS^-/mg	Molar activity equiv.CNS^-/mole
Cysteine	10.0	1.21
Cystine	5.85	1.40
Reduced glutathione	3.70	1.16
Oxidized »	1.48	0.91
» methionine	0	0
Glycine	0	0

serum albumin, 97 equivalent CNS^-/mole (taking 68 000 as the molecular weight for serum albumin). The activity of the disulfide compounds (cystine, oxidized glutathione) was about the same as for the corresponding sulfhydryl compounds (calculated on equimolar basis), but preincubation with cyanide was necessary in this case in order to reach this activity. As one mole of a sulfhydryl compound was formed from one mole of disulfide under these conditions, it was concluded that the activity was due to the sulfhydryl compound formed. In accordance with these findings it was observed that the other reaction product obtained in the cyanolysis of cystine, 2-amino-thiazoline-4-carboxylic acid, was completely inactive. The association of the activity with an intact sulfhydryl groups was demonstrated by blocking the sulfhydryl group in glutathione with iodoacetate, which destroyed the activity, as did oxidation of cysteine to cysteic acid. Other sulfhydryl compounds as sodium sulfide, thioglycolic acid, dithiobiuret and thiophenol were also active, although less than cysteine or glutathione. The catalytic effect of sulfhydryl compounds on the reaction between colloidal sulfur and cyanide might be related to their known ability to react with free sulfur [4]. The end products are a disulfide and hydrogen sulfide in this reaction but probably a persulfide RSSH is initially formed. In the presence of cyanide this persulfide will probably decompose giving thiocyanate and the original sulfhydryl compound. The reaction system can thus be depicted as follows:

(1) $RSH + S \rightarrow RSSH$ followed by
(2) $RSSH + HCN \rightarrow RSH + HCNS$

But at the same time reaction (1) is followed by

(3) $RSSH + RSH \rightarrow RSSR + H_2S$ which is followed by
(4) $RSSR + HCN \rightarrow RSCN + RSH$

Reactions III(3) and (4) can be summarized as

(5) $RSH + S + HCN \rightarrow RSCN + H_2S$.

The active sulfhydryl compound is thus removed from the system through reaction (5) and in accordance with this we observed that the catalytic activity of cysteine decreased with reaction time.

The catalytic effect of serum albumin, on the other hand, was not due to the sulfhydryl groups [5] present in the preparation, as blocking of the latter with N-ethyl-maleimide did not affect the activity. (The complete blocking of the sulfhydryl groups was checked by amperometric titration with mercury

ions according to Kolthoff *et al.*[6]). A preincubation with cyanide in the presence of iodoacetate did not decrease the activity, indicating that the activity was not due to the disulfide groups present in the albumin.

DISCUSSION

The present investigation has only been concerned with rhodanese S from blood serum, although it was previously [2] reported that liver and muscle tissue contains a similar factor. No further experiments have as yet been carried out with these tissues. The catalytic reaction is probably without any physiological significance, as free sulfur has not been demonstrated in tissues from higher animals, and cyanide occurs only in trace amounts [7]. The presence of rhodanese S activity in blood serum may, however, explain the established antidote effect of collidal sulfur in cyanide poisoning [8]. The activity in serum is, as demonstrated in the present work, associated with the serum albumin fraction, and it appears possible to determine the albumin concentration in serum from its rhodanese S activity. A comparative study of the albumin content and rhodanese S activity of different normal and pathological sera is planned. It must, however, be kept in mind that even purified serum albumin preparations have been reported to be inhomogeneous [9], but any attempts at further characterization of rhodanese S in serum albumin have as yet not been made.

The present investigation has not offered any explanation for the catalytic activity of serum albumin. In an attempt to elucidate the reaction mechanism further, the spontaneous reaction between free sulfur and cyanide was studied in a homogeneous system, obtained by replacing water in the standard test system with methanol. The reaction between sulfur and cyanide was then spontaneous and went to completion in less than one minute in confirmation of previous reports in the literature [10]. This may indicate that the effect of the catalyst on the colloidal sulfur-cyanide system is simply due to a facilitation of the contact possibilities between the free sulfur in the solid state and the cyanide.

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Association Equilibria in Solutions of Alkali Salts of Straight Chain Acids. I. Salts of Monocarboxylic Acids

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Association in soap solutions is treated as a reversible chemical reaction. The calculations are based on solubilization data and the effect of an excess of sodium ions on the critical concentration. For the association energy per mole of soap at 20° C an expression $\Delta G_{ass} = 620\ \eta + 3\,680$ cal, is derived where η is the number of methylene groups in the soap molecule.

The association which takes place in solutions of association colloids must be considered a reversible chemical process, and should hence comply with the law of mass action. This necessity has frequently been stressed by investigators, for example, McBain, Hartley, Stauff, Lamm and Hobbs [1-8].

Owing to the fact that very little is known with certainty about the composition and structure of the micelles that are the products of the association, it has been difficult to apply the law of mass action. Also in the formal respect the treatment will be complicated since a very large number of reacting species are involved in micelle formation. Further complications are provided by the stepwise nature of the association process, and by the fact that various equilibria dominate in different concentration ranges.

It is perhaps possible to overcome the last-mentioned difficulties by applying the core and links principle of Sillén [9]. In the following, however, we shall in applying the mass action law to the association process disregard the various intermediate stages of association and consider only the equilibrium between simple ions and the so-called small micelles. One of the main objectives will be to estimate the energy of association per mole of paraffin-chain ion on the basis of available experimental data.

Micelle formation in a soap solution is a reaction of the type:

$$n\mathrm{Me}^+ + m\mathrm{L}^- = (\mathrm{Me}_n^+\ \mathrm{L}_m^-)^{(m-n)-} \qquad (1)$$

where m denotes the number of paraffin-chain ions in the micelle and n the number of counter-ions bound to the micelle. The equilibrium constant for the process is given by the expression

$$K = \frac{a_{mic}}{(a_{Me^+})^n\,(a_{L^-})^m} = \frac{f_{mic}[\mathrm{Me}_n^+\mathrm{L}_m^-]}{f_+^n[\mathrm{Me}^+]^n\,f_-^m[\mathrm{L}^-]^m} \qquad (2)$$

where f_{mic} is the activity coefficient of the micellar particle and f_+ and f_- the activity coefficients of the free ions. The change in free energy per mole, *i. e.* the association energy of associated paraffin-chain ions, ΔG_{ass} will be

$$\Delta G_{ass} = -\frac{RT}{m} \ln \frac{f_{mic}[Me_nL_m]}{f_+^n[Me^+]^n f_-^m[L^-]^m} \quad (3)$$

We shall now attempt to modify this expression by taking into account known experimental data. In the expression the power of f_{mic} is unity and further the concentration of the micellar substance expressed in moles per litre is very low. We may then without any greater error take f_{mic} to be unity.

As Stauff and other investigators have pointed out, important information about the association equilibria is provided by the value of the critical concentration [3]. Ekwall and Harva and later Kolthoff have established that the activities of simple fatty acid ions remain practically constant in the concentration range above the CMC [10, 11]. As we have been able to show, conducted studies of the ability of association colloids to solubilize substances insoluble in water yield further information on the nature of the association process [12]. It is obvious, of course, that the solubilized substance influences the association process, but it is possible to choose for study a sparingly soluble substance which to only a limited extent participates in micelle formation, *i. e.* one that does not appreciably alter the CMC of the association colloid. A suitable compound appears to be *p*-xylene. As we have previously found, the solubilization power of the solution can in this case be considered a measure of the extent of micelle formation [12].

In Fig. 1 the solubility of *p*-xylene is plotted as a function of the sodium laurate concentration in aqueous solution at 20° C [12]. From the curve it is seen that the solubilized hydrocarbon has not appreciably altered the critical concentration, which is 0.026—0.028 M for the pure colloid. At laurate concentrations somewhat above this limit, the solubility curve rises linearly with the soap concentration. The rising linear part of the curve has its origin at a soap concentration of approximately twice the CMC. Also the corresponding plots for other paraffin-chain salts have the same form [12]. The intermediate curved region between the two linear parts is due to the fact that micelle formation is not complete with respect to micelle size and extent of micelle formation immediately above the CMC. A certain higher concentration of the micelle-forming substance is necessary to effect this. Since the concentration of the unassociated soap anion changes relatively little with increase in the total colloid concentration above the CMC, all of the compound introduced at this point must be incorporated in micelles. It is obvious that as soon as the solubilization curve becomes linear and the saturation capacity of the micelles attains a constant value, a concentration range is reached where the properties are largely determined by micelles with a definite composition and structure. [12–16] A similar view of the micelles in this range has been expressed earlier by Hartley and Lamm and quite recently by Stitger, who studied the dependence of the conductance on the colloid concentration [2, 4, 17].

The point where the total colloid concentration is three times the CMC lies in many cases well within the range where the solubility curve is linear

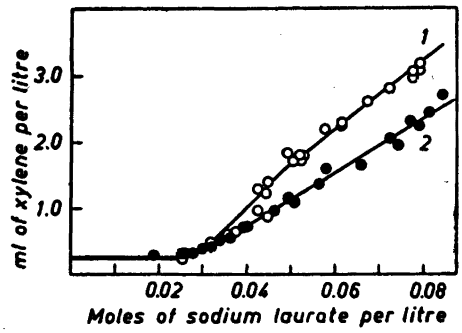

Fig. 1. The solubilization of p-*xylene in sodium laurate at 20° C (curve 1) and 40° C (curve 2). (Ekwall [13].)*

(Fig. 1). We may therefore assume as a first approximation that the concentration of free soap anions is at this point equal to the CMC and that the rest of the soap, whose molecular concentration is twice the CMC, is in the micellar state. Thus when the total colloid concentration is three times the critical concentration, we have

$$[L^-] \approx C_{crit} \qquad (4\ a)$$

and

$$[Me_nL_m] \approx 2\ \frac{C_{crit}}{m} \qquad (4\ b)$$

Before we can make use of this latter expression, we require some knowledge of m. As mentioned above, the saturation capacity of the micellar substance gives some information about the size of the micelles, although strictly speaking the estimate obtained refers to micelles saturated with foreign substance [12]. If however, this substance is one that is incorporated between the palissade layers it is probable that the number of fatty acid ions per micelle is little changed in the solubilization process.

It will be seen below that a variation in m amounting to $\pm$ 50 % has a relatively small effect on the value of ΔG_{ass}. As long as m is greater than 10, the error made when we assume that the number of fatty acid anions is not altered in the solubilization process will not appreciably alter the final result. Studies of the roentgenographic structures, conductances, and other properties of the solutions have indicated that the solubilization of simple cyclic hydrocarbons brings about very small changes in the micelles [18, 19]. On the other hand, according to Booij, straight-chain aliphatic hydrocarbons seem to be at least partly built into the palissade layers [20]. Higher aromatic hydrocarbons possibly also effect marked changes in the micelle structure [21]. Compounds with polar groups participate actively in the formation of the micelles.

It has been found previously that the saturation capacity of oleate micelles (calculated from the linear part of the solubilization curve) for benzene is 186 ml, for toluene 183 ml and for *p*-xylene 177 ml per mole of micellar soap [12]. The volumes per mole of soap are approximately the same for these three substances, whereas when the amounts solubilized are expressed in moles per mole of micellar soap the values vary from 1.44 to 2.99. Similar results have been reported by Klevens [22]. This may be interpreted as evidence for the

assumption that the volumes rather than the number of molecules solubilized are functions of the size of the micelles. In the following we shall assume that the micelles containing no foreign substance contain as many paraffin-chain ions as the micelles that are saturated with xylene.

In order to find the relation between the volume solubilized and the size of the micelle, it is necessary to make certain assumptions as to the structure of the micelle. We shall assume that the soap ions of the micelle form a monomolecular oriented layer around the dissolved hydrocarbon with their ionic groups pointed outwards towards the water and the hydrophobic paraffin-chains directed towards the solubilized hydrocarbon. By assuming the micelles containing hydrocarbon to be spherical in form and the area taken up by a paraffin-chain ion in the palissade layer equal to its cross-sectional area, it is possible to derive the relationship between the saturation capacity and the number of paraffin-chain ions in the micelle [12].

Let

k = saturation capacity of the micellar soap (Å^3 per mole)

q = cross-sectional area of the carbon chain of a soap molecule ($\approx$ 28 Å^2 according to roentgenographic measurements of Harkins [18])

r = radius of the enclosed sphere of xylene

N = Avogadro's number.

The number of paraffin-chain ions necessary to cover the sphere is

$$m = 4\pi r^2/q \qquad (5\ a)$$

The volume of solubilized hydrocarbon is dissolved by m fatty acid ions. It therefore follows that

$$k = 4/3\ \pi r^3 N/m \qquad (5\ b)$$

Eliminating r from equations (5 a) and (5 b), we obtain

$$m = 36\pi k^2/q^3 N^2 \qquad (6)$$

This expression gives the number m of paraffin-chain ions in a micelle as a function of the saturation capacity k. Values of m calculated from this equation for several soaps are given in Table 1. The values calculated are obviously approximate and apply only to swollen micelles, but are of the same magnitude as the values obtained by Debye in a study of the light-scattering of pure solutions of alkyl trimethylammonium bromides and by Hutchinson in a study of sodium alkyl sulphates with alkyl groups of the same length [23, 24]. It is seen that the size of the micelles increases with the length of the paraffin-chain. It is clear that these considerations can apply only in the small micelle range; in higher concentration ranges, the conditions may be quite different [12-16, 25].

We draw attention again to the fact the even a relatively large error in the value of m does not significantly alter the value calculated for the energy of association of a soap molecule. This will be shown below (eq. 9 a).

The value obtained for the energy of association is however greatly dependent on the value assumed for the ratio n/m, the number of counter ions bound in the micelle. The value of the ratio n/m can be calculated from the shift in

Table 1. The saturation capacity (k ml/mole) of the micellar soap for p-*xylene and the number of paraffin-chain ions in soap micelles (m) calculated according to eq. 6* [12].

Soap	Temp.	*k* ml/mole	m
Sodium caprate	20° C	25	10
Sodium laurate	20° C	57	45
Sodium laurate	40° C	42.5	25
Sodium myristate	40° C	70	70
Sodium oleate	20° C	177	440
Sodium oleate	40° C	165	390
Sodium myristyl sulphate	40° C	115	190

the critical concentration caused by added alkali metal ions [26]. Eq. 2 can be written in the form

$$[L^-] = \frac{[Me_n^+ L_m^-]^{1/m}}{K[Me^+]^{n/m}} \qquad (7\ a)$$

Let the total soap concentration be equal to the CMC. If we use the same method to determine the CMC of a soap under various conditions the critical concentration may be defined as the concentration where a certain fraction ε of the soap exists in the micellar state. We may now write equation 7 a in the form

$$(1-\varepsilon)C_{crit} = \frac{(\varepsilon C_{crit})^{1/m}}{m^{1/m} K([Me^+_{tot}]-n/m\varepsilon C_{crit})^{n/m}}$$

Since ε is small compared to 1 and $[Me^+]_{tot} \geqq [L^-]$ in solutions containing excess alkali metal ions, this equation may, after taking logarithms, be written

$$\frac{m-1}{m} \log C_{crit} = -\frac{n}{m} \log [Me^+_{tot}] - K' \qquad (7\ b)$$

When m is large, we obtain the equation given by Corrin

$$\log C_{crit} = -n/m \log [Me^+_{tot}] - K' \qquad (7\ c)$$

Corrin has derived this equation by assuming that the micelles form a separate phase and hence that their activity in the solution remains constant [26].

Using the pinacyanol dye method, Corrin has determined n/m for a large number of paraffin-chain salts. For the potassium salts of fatty acids he found that the value of n/m is almost independent of the chain length, with an average value of 0.56. The present writers have by the same experimental method established that n/m = 0.56 for sodium caprate and 0.64 for sodium laurate at 20° C. The results of our measurements are shown in Fig. 2.

If n/m "gegenions" are bound per soap ion, it is possible to calculate the number of free metal ions at a total colloid concentration of 3 C_{crit} from eqs.

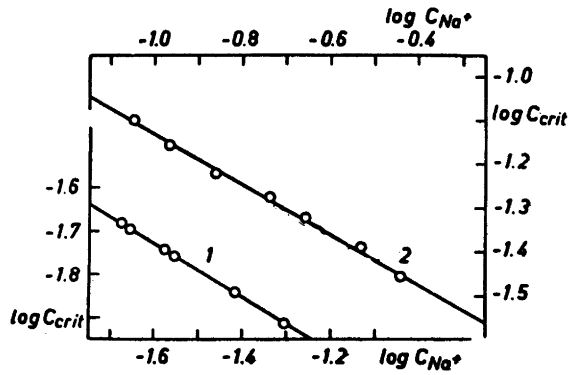

Fig. 2. Curve 1. The shift in CMC of sodium laurate solutions caused by added sodium chloride at 20° C. Curve 2. The shift in CMC of sodium caprate solutions caused by added sodium chloride at 20° C.

4 a and 4 b. The total concentration of metal ions is 3 C_{crit} and the concentration of bound metal ions is according to eq. 4 b equal to n/m 2 C_{crit}. Hence the concentration of free metal ions is

$$[Me^+] = (3—2n/m)\ C_{crit} \tag{8}$$

When the equations 4 a, 4 b and 8 are substituted in 3, we have

$$\begin{aligned} \Delta G_{ass} = {} & RT\ (\ln f_- + \ln C_{crit}) \\ & + RT\ n/m\ [\ln f_+ + \ln (3—2\ n/m) + \ln C_{crit}] \\ & - \frac{RT}{m}\ (\ln f_{mic} + \ln 2\ C_{crit} — \ln m) \end{aligned} \tag{9 a}$$

As already noted, f_{mic} may be taken to be unity. If the colloid in question has a low critical concentration also f_+ and f_- can be set equal to unity. Further, if m is large, *i. e.* the micelles contain more than 10 paraffin-chain ions, the last term can also be omitted. Expression (9 a) will then become

$$\Delta G_{ass} = RT\ (1 + n/m)\ \ln C_{crit} + RT\ n/m\ \ln (3—2\ n/m) \tag{9 b}$$

This equation implies that the value of the association energy is mainly determined by the quantities ln C_{crit} and n/m, provided that the value of m is high. A qualitative interpretation is that the energy required to bring a charged group from the solution to the surface of a micelle with a charge of the same sign is primarily determined by the charge density on the micellar surface and not by the total charge on the micelle.

It is obvious that expressions 9 a and 9 b for the association energy are only approximate. The equations are based on the assumption that the activities of the simple paraffin-chain ions are constant above the critical concentration. This is not fully correct since it has been shown by activity measurements that already in the range below the CMC the activities of simple anions decrease slightly to a value that is definitely lower than the theoretical value[10]. At

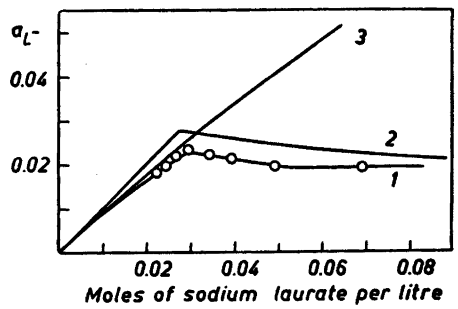

Fig. 3. Curve 1. The activity of laurate ions in sodium laurate solutions at 20° C (Ekwall and Harva [10]). Curve 2. The concentration of laurate ions calculated according to Eq. 7 c. Curve 3. The mean activity of potassium acetate [27].

concentrations above CMC the activity of the simple anions decrease below the value at CMC owing to the fact that the rapid increase in the activity of free alkali ions shifts the association equilibrium in favour of micelle formation. This is clearly indicated by curve 1 in Fig. 3 which shows the variation of the activity of the anion in sodium laurate solutions [10]. For comparison, the variation of the mean activity in sodium acetate solutions is given in curve 3 [27].

It is difficult to interpret the effect described above. By assuming the micelles to be a separate phase in the solution, it is, however, possible to estimate the variation of the anion activity with concentration in a laurate solution. In this case a_{L^-} is assumed to be given by the relation 7 c derived by Corrin [22].

$$\log a_{L^-} \approx - \text{ n/m } \log a_{Me^+} - k' \qquad (7\text{ c})$$

Curve 2 in Fig. 3 gives the activity of the free laurate ion calculated from this equation by assuming all activity coefficients to be unity. It will be noted that curves 2 and 1 in Fig. 3 differ slightly from each other, but are very similar in form.

If we assume that the difference between curves 1 and 2 in Fig. 3 at the concentration 0.030 M is due to the fact that about 10 % of the soap is bound as micelles and that the activities of the simple ions are approximately equal to the activities in potassium acetate solutions containing the same concentration of univalent ions as the soap solution, the application of eq. 7 c gives a curve that very closely follows the experimental values. From this it follows that eq. 7 c gives relatively good values for the activities of the ions in soap solutions.

By taking $f_\pm$ equal to unity and n/m = 0.56, calculation by successive approximation on the basis of eqs. 4 a, 4 b and 7 c, shows that when the total soap concentration is three times the critical concentration $a_{L^-} \approx 0.74\ C_{crit}$. When this value is used to derive a value for the energy of association from 9 a, we obtain after simplification the equation (for 20° C)

$$\Delta G_{ass} = \frac{1\,340}{m} \{\log \frac{m}{2.26} + (m\text{-}1) \log C_{crit}\} + 750 \log C_{crit} \qquad (9\text{ c})$$

All terms of the order of 10 cal were omitted in the derivation of this equation.

Table 2. The association energy (ΔG_{ass}) of the potassium and sodium salts of capric and lauric acid at 20° C calculated according to eqs. 9 a, 9 b and 9 c. The activity coefficients have been taken to be unity.

Soap	C_{crit} mole/litre	m	n/m	$\Delta G_{ass.}$ cal/mole		
				(9 a)	(9 b)	(9 c)
Sodium caprate	0.097	10	0.56	−1 680	−1 920	−1 900
Sodium laurate	0.028	45	0.64	−3 120	−3 200	—
Potassium caprate	0.097	10	0.56	−1 680	−1 920	−1 900
Potassium laurate	0.028	45	0.56	−2 960	−3 050	−3 140

We have calculated from expressions 9 a, 9 b and 9 c the association energies of the sodium and the potassium salts of capric and lauric acids. The values of m employed are those given in Table 1, and have been used for both the sodium and potassium salts. The activity coefficients have been taken to be unity. The results are shown in Table 2. The values obtained for the two laurates differ very little from each other. For the association energies of the caprates, equations 9 a and 9 b give slightly different values. This is due to the low value of m used for the caprates.

It is known that the association energy is determined by the forces of attraction between the hydrocarbon chains in the soap molecules and between these and the water dipoles. These forces are proportional to the number of methylene groups in the carbon chain. On the other hand, repulsive forces act between the ionized groups on the micelle surface. These forces may be assumed to be independent of the length of the hydrocarbon chain and hence remain constant at least as long as the charge density on the micelle surface does not change. The charge density will remain the same if the ratio n/m remains constant. From these observations it follows that if we determine the association energies for a series of homologues and plot these as a function of the number of methylene groups, a linear plot $\Delta G_{ass} = a + \eta b$ should result, where η is the number of methylene groups in the paraffin-chain. The slope b gives the association energy per methylene group. The value b obtained on the basis of the calculated association energy of potassium laurate and caprate at 20° C is —620 cal per methylene group. This value is in satisfactory agreement with the value 1.08 RT derived by Shinoda [6]. The intercept a is + 3 680 cal at 20° C according to the values in Table 2. This energy is required to incorporate a charged carboxyl group in the micelle surface. It then follows that for the potassium soaps the association energy is

$$\Delta G_{ass} = -620\eta + 3\,680 \text{ cal} \qquad (10)$$

It is here assumed, of course, that the hydrocarbon-chain is straight.

It is difficult to decide whether these values are correct. The energy liberated in the association is too small to be measured by calorimetric methods. In order to determine whether our reasoning is valid, we have calculated the association energy for several soaps from eq. 10. These values are given in the

Table 3. The association energy ΔG_{ass} and CMC of various soaps calculated on the basis of eq. 10 and eqs. 9 b and 9 c compared with experimental values of the CMC.[12,28,29,30]

Soap	$\Delta G_{ass.}$ cal./mole	CMC mole/litre	
		calc.	found
Potassium hexanoate	+ 580	1.5	1.5
—»— heptanoate	— 40	0.78	0.78
—»— octanoate	— 660	0.38	0.39
—»— nonanoate	—1 280	0.20	0.20
—»— undecanoate	—2 520	0.05	0.049
—»— tridecanoate	—3 760	0.012	0.0126
—»— tetradecanoate	—4 380	0.0075	0.0066
Sodium hexadecanoate	—5 620	0.0017	0.0032
—»— octadecanoate	—6 860	0.0004	
—»— oleate	—6 860	0.0004	0.0017—0.0008

second column of Table 3. The third column in Table 3 gives the critical concentrations of the soaps calculated from eq. 9 c for those cases for which the value of m can be estimated from available data. When the value of m was not known, the CMC was calculated from eq. 9 b. Experimentally determined values for the CMC are given in the fourth column. The agreement is satisfactory. Actually the values of Table 3 do not present anything new, for they only confirm the established logarithmic relationship between CMC and the length of the hydrocarbon chain [3].

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Association Equilibria in Solutions of Alkali Salts of Straight Chain Acids. II. Salts of Monocarboxylic Acids

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The values calculated for the association energy in Part I are applied to the association process that takes place in solutions of the potassium salts of α,ω-alkanedicarboxylic acids[1]. The critical concentrations of these salts are calculated from the values derived for the energy of association and are compared with experimentally determined values.

In Part I it was shown that the energy of association per mole of a straight-chain monocarboxylic soap can be computed from the equation

$$\Delta G_{ass} = -620\,\eta + 3\,680 \text{ cal.} \qquad (10)$$

where η is the number of methylene groups in the soap molecule [1].

It should be possible to extend the same equation to the association of potassium salts of α,ω-alkanedicarboxylic acids. Obviously it is necessary to double the electrical work involved in the association process because of the presence of two charged carboxylate groups in the molecule. We then obtain

$$\Delta G_{ass} = -620\,\eta + 7\,360 \text{ cal.} \qquad (11)$$

where η is again the number of methylene groups in the molecule. Values for the association energies calculated from this equation for a number of alkane dicarboxylic salts are given in Table 1.

Table 1. The association energies of potassium salts of α,ω-alkanedicarboxylic acids at 20° C calculated according to eq. 11.

Salt	ΔG_{ass} cal./mole
KOOC—$(CH_2)_8$—COOK	+2 400
KOOC—$(CH_2)_{11}$—COOK	+ 540
KOOC—$(CH_2)_{14}$—COOK	−1 320
KOOC—$(CH_2)_{16}$—COOK	−2 560

Qualitatively these values are in accordance with known properties of these compounds [2]. The potassium salt of decanedioic acid does not undergo association in aqueous solution, while in the case of the potassium salt of tridecanedioic acid, signs of association are observed only in very concentrated solutions. Association in solutions of the potassium salt of hexadecanedioic acid occurs when the concentration exceeds 0.4 molal, but the potassium salt of the octadecanedioic acid is a typical association colloid with a critical concentration of 0.05 molal [2].

The association energy values in Table 1 can be employed to calculate the critical concentrations of the dicarboxylic salts. The micelle formation by these salts can be treated in the same manner as in the case of normal soaps, with the difference, however, that in the application of the mass action law, two alkali ions are associated with each dicarboxylate ion. Also the number of "gegenions" bound to each dicarboxylate ion is twice the number in the case of normal soaps. In the following it is assumed that the average number of potassium ions bound is $n/m = 2 \times 0.56 = 1.12$ moles per mole salt.

We shall again choose as the starting point in our treatment the total concentration equal to three times the critical concentration. A first approximation based on eqs. 1 — 4 b in Part I [1] is that at this total concentration the concentration of unassociated paraffin-chain anions is C_{crit} and the quantity of anions bound in the micelles is 2 C_{crit}. The total alkali ion concentration is 6 C_{crit}, the quantity of bound cations is 2.24 C_{crit} and that of free cations is hence 3.76 C_{crit}.

The free alkali ions should decrease the activity of the fatty acid ions by promoting association in accordance with expression 7. By applying successively eqs. 1 — 4 b and 7, it is found that at a total concentration 3 C_{crit} the activity of the free dicarboxylate ions is approximately 0.57 C_{crit}, that of the free alkali ions is 3.28 C_{crit}, and the concentration of the micelles is $1/m \times 2.43\ C_{crit}$. By substituting these values in the expression 3 for the association energy, we find that at 20° C

$$\Delta G_{ass} = 1\,340\ (2.12-1/m)\ \log C_{crit} + 1\,340/m\ \log\ (m/2.43)+450 \text{ cal.} \quad (12)$$

According to this equation the free energy change will be largely determined by m when this has a low value. For this reason it is necessary to know the order of magnitude of m. As in the case of normal soaps, an approximate value for m can be estimated on the basis of the solubilizing power of the micelles.

Solubilization data for the potassium salts of alkanedicarboxylic acids suggest that these salts form single-layered micelles [2]. There is reason to assume that the micellar aggregates have all three dimensions equal, as in the dilute solutions of ordinary soaps. From roentgenographic data is known that the increase in length per methylene group in a hydrocarbon-chain is 1.25 Å and the cross-sectional area taken up by the hydrocarbon-chain in a micelle [3] is about 28 Å^2. It hence follows that if a micelle of the potassium salt of tridecanedioic acid has equal dimensions in all directions, the micelle will contain approximately eleven dicarboxylate ions. In a corresponding manner a KOOC—$(CH_2)_{14}$—COOK micelle can be estimated to contain 17 and a KOOC—$(CH_2)_{16}$—COOK micelle 22 ions.

Table 2. The association energies of the potassium salts of a,w-alkanedicarboxylic acids at 20° C calculated according to eq. 12.

Salt	ΔG_{ass} cal./mole
$KOOC-(CH_2)_{11}-COOK$	2 710 log C_{crit} + 540
$KOOC-(CH_2)_{14}-COOK$	2 750 log C_{crit} + 520
$KOOC-(CH_2)_{16}-COOK$	2 770 log C_{crit} + 510

When these values of m are substituted in eq. 12 the values in Table 2 are obtained for the changes in the free energies in the association.

It is now possible to calculate the critical concentrations by comparing the values of ΔG_{ass} in Table 1 and Table 2. The value of C_{crit} obtained for $KOOC-(CH_2)_{11}-COOK$ is 1.0 molal. The experimental value obtained [2] from a study of the solubilization of *n*-decanol is 1.8 molal at 40° C. The calculated and experimental values for $KOOC-(CH_2)_{14}-COOK$ are 0.22 molal and 0.4 molal at 40° C, respectively. The agreement is satisfactory in view of the low value of the activity coefficient in the concentrated solutions involved. For $KOOC-(CH_2)_{16}-COOK$ the calculated critical concentration is 0.075 molal and the experimental value [2] based on conductivity and solubilization power is 0.05 molal at 60° C.

As already pointed out, it is difficult to confirm the values derived for the free energy change. It seems, however, that the values derived for the free energy change can be used to determine whether the paraffin-chain salt undergoes association or not and to evaluate approximately the concentration where association begins.

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Synthèse des Esters Acides d'Acides Dicarboxyliques par Action de l'Alcoolate de Sodium ou de Potassium sur les Anhydrides d'Acide Correspondants

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Une méthode pour préparer des esters acides des acides dicarboxyliques qui sont capables de former des anhydrides cycliques est décrite et illustrée par la préparation de 24 esters de 7 différents acides dibasiques.

On discute l'influence d'un substitution non-symétrique dans l'anhydride d'acide et l'influence du radical de l'alcool sur le choix de la fonction carboxylique estérifiée.

On a étudié, à plusieures reprises, la synthèse des esters acides des acides dicarboxyliques par réaction entre l'anhydride d'acide et l'alcool, et, par exemple pour l'acide phthalique et l'acide succinique, l'ébullition de l'anhydride d'acide avec l'alcool est indiquée comme la meilleure méthode pour la synthèse des esters acides. Pour des autres acides dicarboxyliques, par contre, assez souvent les esters acides ne sont pas connus à l'état de pureté, la formation de l'ester acide étant accompagnée de la formation de l'ester neutre.

La réaction entre un anhydride d'acide et un alcool peut être catalysée par des bases de sorte qu'un anhydride d'acide, dissous dans un alcool, est transformé en ester acide par addition d'une quantité équivalente de l'alcoolate de sodium. Cette réaction a été étudiée par exemple par Zelinski [1], Walker [2], Cazeneuve [3,4], Brühl et Braunschweig [5,6], Hoogewerff et van Dorp [8], Wegscheider [8,9,10,11] et Mol [12], mais il semble que l'utilisation pour la synthèse des esters acides a été assez limitée, peut être parce que, dans le cas des anhydrides des acides dicarboxyliques non symétriques, il peut se former un mélange des deux esters acides différents.

L'application analytique pour le dosage des anhydrides d'acides mono- ou dicarboxyliques a, par contre, été étudiée d'une facon plus détaillée, plus récemment par Nicolas et Burel [13], et l'on a trouvé que généralement non seulement les anhydrides d'acides sont ouverts par le méthylate de sodium, mais aussi les imides sont transformées semblablement, en tous cas par application

d'un solvant anhydre comme le butylamine (Fritz et Lusche [14]). Les lactones, par contre, ne réagissent que si elles sont trés facilement hydrolysées par l'eau (Smith et Bryant [15]). Il faut remarquer, toutefois, que les azlactones sont alcoolysées très rapidement par l'alcoolate de soude (voir Baltazzi [16]).

Comme nous avons trouvé que quelques lactames cycliques sont transformées en esters des acides correspondants par addition de la soude aqueuse décinormale à une solution éthanolique de ces lactames (ces expériences seront publiées dans une autre communication), nous nous sommes proposés d'examiner l'application synthétique de la réaction entre des anhydrides d'acides et un alcoolate de sodium ou potassium.

Voici le procédé dont nous nous sommes servis:

0.1 mol. d'anhydride d'acide est dissous ou mis en suspension dans 50—100 ml de l'alcool dont on pense préparer l'ester acide. Une solution de 2.3 g de sodium ou 3.9 g de potassium (0.1 atome) dans 50—100 ml du même alcool ou 100 ml d'une solution de soude ou de potasse (1 N) dans cet alcool est placée dans un entonnoir à robinet et ajoutée goutte à goutte pendant 30—45 minutes à la solution de l'anhydride d'acide sous agitation mécanique ou à la main. L'agitation est continuée encore 15 minutes. L'excès d'alcool est alors enlevé, au bain-marie dans le cas du méthanol, dans le vide dans le cas d'un alcool avec point d'ébullition plus élevé, en ayant soin que la température dans le matras n'excède pas 150° pour éviter le dédoublement du sel de l'ester acide en un sel de l'acide et l'ester neutre de l'acide.

L'alcool enlevé, le sel de l'ester acide est recristallisé p. ex. dans un mélange d'éthanol et d'acétone.

Très souvent il est préférable d'isoler le sel de l'ester acide par précipitation avec l'éther sans enlever l'alcool.

Pour libérer l'ester acide, le sel est dissous dans l'eau et 0.1 mol. d'acide chlorhydrique dilué est ajouté. L'ester acide libéré est isolé par filtration dans le cas des esters solides, à l'aide d'un entonnoir à robinet dans le cas des esters liquides à la temperature ambiante. Si l'ester acide est soluble dans l'eau on peut l'isoler par extraction avec l'éther.

Les esters solides sont recristallisés dans le méthanol dilué, dans la ligroine (eb. 100—140°) ou dans un mélange de solvants appropriés.

On ne peut pas purifier les esters acides liquides par distillation, car d'après l'indication d'Anschütz [17] les esters acides se dédoublent en ester neutre et acide libre ou anhydride d'acide par distillation, même dans le vide.

Pour la préparation des sels des esters acides, on peut, au lieu d'ajouter une solution de l'alcoolate de sodium dans l'alcool, se servir d'une solution aqueuse de soude, 1 N, ajoutée goutte à goutte à une solution de l'anhydride d'acide dans l'alcool. Par ce procédé, il se forme un mélange du sel neutre de l'acide, du sel de l'ester acide et de l'acide libre, le rendement de sel de l'ester acide étant souvent 70—80 %.

Ce résultat doit être étudié en le comparant avec les résultats obtenus par Tirouflet [18] et par Véne et Tirouflet [19], qui ont étudié l'hydrolyse alcaline des phthalides et des anhydrides phthaliques substitués. Ils ont déterminé la cinétique de l'ouverture des phthalides et des anhydrides phthaliques, d'une part dans un milieu aqueux et d'autre part dans un milieu alcoolique-aqueux. Ils trouvent que plus la concentration de l'alcool est grande, plus grande est

la vitesse de la solvolyse, mais ils ne semblent pas se rendre compte que l'alcoolyse peut être manifeste même en un milieu assez riche en eau, et ils n'ont pas déterminé la vitesse de la solvolyse dans un milieu purement éthanolique.

Au point de vue préparatif, ce sont les anhydrides des acides symétriques qui sont les plus utiles, car ils ne permettent qu'un produit de réaction, tandis qu'avec les anhydrides des acides dibasiques sans symétrie généralement deux produits de réaction sont formés, et la séparation des deux esters acides isomères n'est pas toujours possible. D'autre part, si l'on réussit à séparer les deux isomères, l'étude des anhydrides d'acides sans symétrie présente une possibilité d'étudier l'influence du caractère de donneur ou d'accepteur d'électrons d'un substituant quelconque sur la vitesse d'estérification et de la comparer avec l'influence du substituant sur pK_{S_1} de l'acide dibasique correspondant. De même, on peut étudier l'influence de la structure de l'alcool sur la vitesse d'estérification et sur le point d'attaque de l'ion alcoolate pour voir si la rupture des liaisons dans l'anhydride cyclique a toujours lieu sur le même côté de l'atome d'oxygène ou si p. ex. les ions de méthylate et les ions de benzylate vont attaquer deux côtés différents de l'atome d'oxygène.

Les expériences décrites ci-dessous laissent soupçonner que plus le caractère électrophile du substituant est fort, plus la rupture de l'anhydride cyclique est sélective, de sorte que dans le cas des anhydrides citraconique et phénylsuccinique, deux esters acides sont formés, tandis que dans le cas de l'anhydride chloromaléinique, nous n'avons trouvé qu'un des esters acides isomères.

Nos résultats s'écartent donc de la règle mentionnée par Salmon-Legagneur dans le Traité de Chimie Organique de V. Grignard [20]: "Lorsque l'on part de l'anhydride d'un diacide ayant un carbone substitué en α ou α', on obtiendra, en règle générale, à l'exclusion de l'autre isomère possible, l'éther dont la fonction acide non éthérifiée est la moins énergique (c'est à dire celle rattachée au carbone le plus substitué). Comme le fait remarquer Blaise [21] l'étherification l'emporte sur la salification; la méthode marche donc de pair avec le procédé à l'étherification partielle et complète celui basé sur la demi-saponification."

En ce qui concerne l'influence du radical des ions d'alcoolate il semble qu'avec les ions méthylates, la fonction carboxylique la plus proche du substituant est estérifiée, avec les ions de benzylate, par contre, c'est parfois l'autre fonction carboxylique (empêchement stérique?).

Ces deux questions doivent, toutefois, être examinés plus à fond que nous ne l'avons fait ici pour que l'on puisse en déduire des règles générales.

PARTIE EXPÉRIMENTALE

Acide succinique

a. Succinate acide de méthyle. Pour obtenir le succinate acide de méthyle à l'état de pureté il n'est pas besoin d'isoler le sel de sodium. Voici un procédé qui a donné l'ester cherché dans un rendement de 69 %.

A une solution de 10.0 g (0.1 mol.) d'anhydride succinique dans 100 ml de méthanol fut ajouté goutte à goutte sous agitation mécanique une solution de 2.3 g (0.1 atome) de sodium dans 100 ml de méthanol. Après un repos de 15 minutes, on ajoute 100 ml

d'acide chlorhydrique 1.0 N, en refroidissant la solution dans l'eau glacée. Le chlorure de sodium précipité fut enlevé par filtration, le filtrat évaporé dans le vide (température du bain 40°), le résidu dissous dans l'éther pour séparer l'ester d'une petite quantité d'acide succinique libre et des traces de chlorure de sodium. Par évaporation spontanée de l'éther, le succinate acide de méthyle cristallise. Rendement 9.1 g ou 69 %. F 59—60°, E, poids d'équivalent, (titration avec la soude) 131.4, calculé 132.1.

Si, au lieu de méthylate de sodium, on ajoute une solution d'hydroxyde de potassium dans méthanol, 1 N, on obtient, à côté du sel de potassium de l'ester acide, et le succinate neutre de potassium et l'ester acide libre. Ce dernier peut former un sel double avec son sel de potassium. En ajoutant 100 ml de la solution méthanolique de l'hydroxyde de potassium à 10 g d'anhydride succinique dissous dans 100 ml de méthanol et en évaporant la solution au bain-marie jusqu'à 40 ml, on obtient en refroidissant le résidu dans l'eau glacée 3 g d'un sel de potassium dont on trouve pour le poids d'équivalence par titration avec la soude 305, par titration avec l'acide perchlorique dans l'acide acétique cristallisable 307, c'est à dire que le sel contient des nombres égaux des fonctions carboxyliques libres et neutralisées, les prémières étant déterminées par titration avec la soude, les dernières par titration avec l'acide perchlorique. Une analyse élementaire corréspond à la formule $CH_3OCO-CH_2-CH_2-COOK$, $CH_3OCO-CH_2-CH_2-COOH$; M_{calc} 302.3; trouvé C 39.92; H 5.28. Calc. ($C_{10}H_{15}O_8K$) C 39.73; H 5.00.

b. Succinate acide d'isopropyle. Une solution de 3.9 g (0.1 atome) de potassium dans 50 ml d'isopropanol fut ajouté à une solution de 10 g (0.1 mol) d'anhydride succinique dans 25 ml d'isopropanol. Le sel de potassium fut précipité par 500 ml d'éther. Rendement 14.8 g ou 75 %. Titré avec l'acide perchlorique dans l'acide acétique cristallisable le sel a montré E = 190; calc. E = 198.3, c'est à dire que le sel brut contient une petite quantité du sel neutre de l'acide succinique.

14 g du sel de potassium fut transformé en succinate acide d'isopropyle en acidifiant la solution aqueuse avec l'acide chlorhydrique dilué. L'ester acide fut isolé par extraction avec l'éther, la solution éthérée séchée sur sulfate de sodium anhydre et le solvent enlevé dans le vide. Le résidu fut recristallisé dans un mélange d'éther et d'éther de pétrole. Rendement 8 g ou 70 %; calculé à partir de l'anhydride succinique le rendement est 50 %. E (par titration avec la soude) 159; calc. 160.2. F 50—51°. Hückel, Kumetat, Ullmann et Doll [22] indiquent F 51°.

c. Succinate acide de benzyle. Le sel de sodium de l'ester-acide fut préparé comme indiqué ci-dessus pour l'ester méthylique, substituant l'alcool benzylique pour l'alcool méthylique. L'alcool benzylique fut enlevé dans le vide, température du bain 125—130°, température des vapeurs 100° au maximum. Le résidu fut dissous dans l'eau; par acidification une huile se sépare qui, après refroidissement dans l'eau glacée et amorçage avec succinate acide de benzyle cristallise. Rendement 90—95 %; après recristallisation dans la ligroine (Éb. 100—140°) 80 % avec F 60—61° (littérature 59°).

d. Succinate acide de cyclohexyle. On a essayé de préparer cet ester-acide par un procédé analoque, mais sans succés. Il a été seulement obtenu un rendement faible en un sel très impur, impossible à purifier par recristallisation.

Acide maléique

Des esters acides de l'acide maléique ont été préparés par des procédés plus compliqués que celui que nous venons de décrire, et le plus souvent la pureté des esters acides obtenus n'a pas été suffisante. Le maléate acide de méthyle a été préparé dans l'état de pureté par Walker [2], qui indique pour cet ester-acide $pK_A^{25°} = 1.1 \cdot 10^{-3}$. (L'acide maléique $pK_{S_1} = 4 \cdot 10^{-2}$). Anschütz [17] a préparé le maléate acide d'éthyle sous forme d'huile, pour laquelle il indique une analyse élementaire satisfaisante. La préparation de plusieurs autres esters acides a été décrite, mais aucun de ces esters n'ont été isolés dans l'état de pureté.

a. Maléate acide de méthyle. Le sel de sodium de cet ester fut préparé par le procédé décrit plus haut et isolé par évaporation de l'alcool méthylique sur bain-marie. Le résidu fut dissous dans 50 ml de méthanol, la solution filtrée et le sel de sodium précipité par addition de de 250 ml d'éther et isolé par filtration après refroidissement dans l'eau glacée. E (titration avec l'acide perchlorique) 169; calc. 170.1. Rendement (partant de

10 g d'anhydride maléique) 13.5 g ou 80 %. Trouvé C 36.13; H 4.37; Na 13.87. Calc. ($C_5H_5O_4Na$, H_2O): C 35.32; H 4.15; Na 13.53. Le sel de sodium cristallise donc avec 1 mol d'eau.

Le sel de potassium fut préparé en ajoutant lentement (agitation à la main) une solution de 3.9 g (0.1 atome) de potassium dans 25 ml de méthanol à une solution de 9.8 g (0.1 mol) d'anhydride maléique dans 25 ml de méthanol, le mélange s'échauffant faiblement pendant l'addition. Après un repos d'une heure, 500 ml d'éther furent ajoutés, précipitant une substance incolore qui après refroidissement dans la glacière fut isolée par filtration, lavée avec l'éther et séchée dans le vide sur l'acide sulfurique. Rendement 15.3 g ou 92 %. E (titration avec l'acide perchlorique) 166; calc. 168.2.

Le sel fut recristallisé deux fois dans un mélange d'éthanol et acétone. Trouvé C 35.75; H 3.23. Calc. ($C_5H_5O_4K$): C 35.71; H 2.98. Le sel de potassium est donc anhydre.

L'ester-acide fut isolé par acidification d'une solution aqueuse du sel de potassium et extraction de la solution avec l'éther. La solution éthérée fut séchée sur sulfate de sodium anhydre, le solvant enlevé par évaporation, laissant une huile qui ne cristallise pas à $-10°$.

b. Maléate acide d'éthyle. Le sel de potassium de cet ester-acide fut préparé sur le modèle de l'ester méthylique. Rendement 90 %. E du produit brut (titration avec l'acide perchlorique) 175, calc. 182.2. Après deux recristallisations dans un mélange d'éthanol et acétone on trouve E 182; C 39.35; H 4.07. Calc. ($C_6H_7O_4K$): C 39.54; H 3.88.

L'ester-acide fut isolé comme l'ester-acide méthylique, formant une huile qui ne cristallise pas à $-10°$.

c. Maléate acide d'isopropyle. Sur le même modèle le sel de potassium du maléate acide d'isopropyle fut isolé avec un rendement de 82 %. E du produit brut (titration avec l'acide perchlorique) 196, calc. 196.3, avec 1/2 mol d'eau 205.3. Après deux recristallisations dans un mélange d'éthanol et d'éther on trouve E 206; C 41.28; H 5.06. Calc. ($C_7H_9O_4K$, 1/2 H_2O): C 41.00; H 4.92.

L'ester-acide fut isolé comme ci-dessus, formant une huile qui ne cristallise pas à $-10°$.

d. Maléate acide de cyclohexyle. 3.9 g (0.1 atome) de potassium furent dissous dans 100 ml de cyclohexanol. La solution fut chauffée à 50° et ajoutée sous agitation à la main à une suspension de 9.8 g (0.1 mol) d'anhydride maléique dans 50 ml de cyclohexanol, chauffant le mélange de temps en temps pour éviter une cristallisation du cyclohexylate de potassium. Après un repos d'une heure aucune précipitation n'a lieu par refroidissement. On précipite alors le sel de potassium de l'ester-acide par addition d'éther, on l'isole à la trompe, on le lave avec l'éther et le sèche dans le vide sur acide sulfurique.

Recristallisé deux fois dans un mélange de méthanol et acétone le rendement fut 17.7 g ou 75 %. E (titration avec l'acide perchlorique) 236, calculé 236.3.

5 g du sel de potasse furent dissous dans 15 ml d'eau, la solution aqueuse fut acidifiée avec l'acide chlorhydrique dilué et extraite avec 3 × 25 ml d'éther éthylique. La solution éthérée fut séchée sur sulfate de sodium anhydre, l'éther enlevé par évaporation et le résidu séché dans le vide sur acide sulfurique, se prenant en masse par ce traitement. Rendement 2.8 g ou 67 % avec F 63—64° et E (titration avec la soude) 197; calc. 198.2.

Recristallisé deux fois dans l'éther de pétrole (Eb. 60—100°) on trouve F 63.5—64.5°; C 60.55; H 7.19. Calc. ($C_{10}H_{14}O_7$): C 60.61; H 7.07.

e. Maléate acide de benzyle. Une solution de 3.9 g (0.1 atome) de potassium dans 100 ml d'alcool benzylique fut ajoutée sous agitation à la main à une solution de 9.8 g (0.1 mol) d'anhydride maléique dans 100 ml d'alcool benzylique. Après un repos d'une heure, pendant lequel le mélange fut agité de temps en temps, le sel de potassium fut précipité par addition d'un litre d'éther. Après refroidissement dans l'eau glacée, le sel fut isolé à la trompe, lavé avec l'éther et séché. Rendement 20.3 g ou 82 %. E (titration avec l'acide perchlorique) 243, après une recristallisation dans l'éthanol 245,2; calc. 244,3.

10 g du sel de potassium furent dissous dans 20 ml d'eau, la solution acidifiée avec l'acide chlorhydrique et extraite avec 3 × 25 ml d'éther éthylique. La solution éthérée fut séchée sur sulfate de soude anhydre, l'éther enlevé par évaporation et le résidu séché dans le vide sur acide sulfurique, se prenant en masse dans l'exsiccateur. Recristallisé dans un mélange d'éther et d'éther de pétrole (Eb. 60—100°) le rendement fut 7 g ou 83 % avec F 53—54°, après encore une recristallisation 54—55°; E (titration avec la soude) 206; C 63.90; H 4.87. Calc. ($C_{11}H_{10}O_4$): E 206.2; C 64.03; H 4.89.

Acide glutarique

a. Glutarate acide de méthyle. Une solution de 1 g (0.025 atome) de potassium dans 10 ml de méthanol fut ajoutée à une solution de 2.85 g (0.025 mol) d'anhydride glutarique dans 10 ml de méthanol. Après une heure de repos le sel de potassium fut précipité par 300 ml d'éther. Rendement 4.3 g ou 93 %. E (titration avec l'acide perchlorique) 184; calc. 184.2.

Le sel fut recristallisé deux fois dans un mélange de méthanol et acétone. Trouvé C 38.55; H 5.02. Calc. ($C_6H_9O_4K$): C 39.12; H 4.93. Le sel est très hygroscopique, ce qui explique la valeur faible trouvée pour C.

L'ester-acide fut isolé sur le modèle des esters-acide maléiques, formant une huile qui ne cristallise pas à $-10°$. Fourneau et Sabetai [23] l'ont préparé par réaction entre l'acide glutarique et le glutarate de méthyle, Clutterbuck et Raper [24] par réaction entre l'anhydride glutarique et le méthanol.

b. Glutarate acide d'isopropyle. Préparé sur le modèle habituel le sel de potassium fut obtenu avec un rendement pauvre. La purification est difficile, car le sel est extrèmement hygroscopique.

Recristallisé 3 fois dans un mélange de chloroforme et éthanol absolu on trouve E (titration avec l'acide perchlorique) 214; C 44.70; H 6.32. Calc. ($C_8H_{13}O_4K$): E 212.3; C 45.30; H 6.18.

L'ester-acide libre ne fut pas isolé à l'état de pureté.

c. Glutarate acide de benzyle. Une solution de 1 g (0.025 atome) de potassium dans 10 ml d'alcool benzylique fut ajoutée à une solution de 2.85 g (0.025 mol) d'anhydride glutarique dans 10 ml d'alcool benzylique. Le sel de potassium fut précipité par 250 ml d'éther. Rendement brut 5.8 g ou 88 %, qui 2 fois recristallisés dans l'éthanol absolu ont donné 1.25 g ou 19 % du sel pur. Trouvé E (titration avec l'acide perchlorique) 260; C 55.10; H 5.17. Calc. ($C_{12}H_{13}O_4K$): E 260.3; C 55.36; H 5.03.

L'ester-acide fut isolé, mais seulement comme un produit brut avec F ca. 25°.

d. Il faut ajouter que Mol [12] a préparé le *glutarate acide d'éthyle* avec un rendement à peu près quantitatif par réaction entre l'anhydride glutarique et l'éthylate de sodium.

Acide phthalique

a. Phthalate acide de méthyle. Une solution de 5 ml d'hydroxyde de sodium 10 N (0.05 mol) dans 150 ml de méthanol fut ajoutée à une solution de 7.4 g (0.05 mol) d'anhydride phthalique dans 400 ml de méthanol au cours d'une demi-heure sous agitation mécanique. Après un repos de 15 minutes 12.5 ml acide chlorhydrique (4 N) furent ajoutés et le mélange évaporé dans le vide (température du bain 40—50°). Le résidu fut recristallisé dans 50 ml de ligroine (Éb. 100—140°) pour séparer l'ester-acide du chlorure de sodium. Rendement 7.6 g ou 85 %. F après encore une recristallisation 84° comme indiqué dans la littérature.

On peut préparer l'ester-acide en milieu plus aqueux que celui indiqué ici, mais l'ester-acide est alors mélangé avec l'acide libre, et la purification est assez compliquée. A une solution de 7.4 g (0.05 mol) d'anhydride phthalique dans 100 ml de méthanol furent ajoutés 50 ml soude (N) pendant 1/2 heure sous agitation mécanique. Après un repos d'une demi-heure 12.5 ml d'acide chlorhydrique (4 N) furent ajoutés. L'ester-acide fut isolé comme ci-dessus; rendement 7.35 g ou 83 % qui contient encore de l'acide phthalique, car par titration avec 0.1 N soude on trouve E 160; calculé pour l'ester-acide E 180, pour l'acide phthalique E 83, c'est à dire que le produit contient 80 mol % ou 90 % par poids de l'ester-acide.

b. Phthalate acide d'éthyle a été préparé avec un rendement à peu près quantitatif en mélangeant une solution éthanolique de l'anhydride phthalique avec une solution d'éthylate de sodium (voir Zelinski [1]).

c. Phthalate acide d'isopropyle. Une solution de 2 g (0.05 atome) de potassium dans 25 ml d'isopropanol fut ajoutée à une solution de 7.4 g (0.05 mol) d'anhydride phthalique dans 25 ml d'isopropanol. Après un court repos le sel de potassium fut précipité par 500 ml d'éther éthylique, filtré à la trompe, lavé avec l'éther et séché dans le vide sur acide sulfurique. Rendement 11 g ou 90 %. E (titration avec l'acide perchlorique) 247; calc. 246.3.

Une solution de 5 g du sel de potassium dans 25 ml d'eau fut acidifiée avec l'acide chlorhydrique dilué et extrait par 3 × 25 ml d'éther. La solution éthérée fut séchée sur sulfate de sodium anhydre et l'éther enlevé par évaporation (bain-marie). Le résidu cristallise en ajoutant l'éther de pétrole. Rendement 3.5 g ou 83 %. F 78—80° qui reste constant après deux recristallisations dans ligroine. Trouvé E (titration avec la soude) 206; C 63.25; H 5.90. Calc. ($C_{11}H_{18}O_4$): E 208.2; C 63.45; H 5.81.

Le phthalate acide d'isopropyle a été préparé par Morin et Bearse [25] qui ne donnent pas le F de la substance.

d. Phthalate acide de butyle. A une solution de 7.4 g (0.05 mol) d'anhydride phthalique dans 100 ml de butanol furent ajoutés 50 ml hydroxyde de sodium (N) pendant 10 minutes sous agitation mécanique, qui est prolongée encore une demi-heure. Le mélange est concentré dans le vide (température du bain 30—40°) jusqu'à 20 ml. Le résidu est soluble dans l'eau. La solution aqueuse est extraite par l'éther pour enlever les derniers restes de butanol et acidifiée avec 12.5 ml d'acide chlorhydrique 4 N. Une huile se sépare qui est extraite par l'éther. La solution éthérée est séchée sur sulfate de sodium anhydre et l'éther enlevé par évaporation, laissant un résidu cristallin. Rendement 8.1 g ou 73 % avec F 74.5—76°, après recristallisation dans l'éthanol dilué F 75—76° (Goggans et Copenhaver [26] indiquent F 73.1—73.5°).

e. Phthalate acide de cyclohexyle. Le sel de potassium fut préparé sur le modèle du sel de potassium du phthalate acide d'isopropyle. Rendement 97 %. E trouvé (titration avec l'acide perchlorique) 292; calc. 286.4.

Pendant la recristallisation du sel dans l'éthanol dilué, il se décompose.

L'ester-acide fut préparé à partir du produit brut du sel. Nous l'avons obtenu comme une huile, qui ne cristallise pas, quoique Brunel [27] en indique F 99°.

Acide citraconique

a. Citraconate acide de méthyle. A une solution de 5.6 g (0.05 mol) d'anhydride citraconique dans 25 ml de méthanol fut ajoutée une solution de 1.95 g (0.05 atome) de potassium dans 25 ml de méthanol. Après un repos d'une heure le mélange fut évaporé dans le vide, laissant une huile qui, couverte de 250 ml d'éther et laissée à la glacière à —10° jusqu'au lendemain, cristallise. Rendement après recristallisation dans un mélange d'éthanol et d'éther 8.3 g ou 91 %. E (titration avec l'acide perchlorique) 184; calc. 182.2. Après encore deux recristallisations dans un mélange de méthanol et d'acétone on trouve E 182; C 39.44; H 3.98. Calc. ($C_6H_7O_4K$): E 182.2; C 39.54; H 3.87.

Une solution aqueuse de 5 g du sel de potassium fut acidifiée et extraite avec l'éther. La solution éthérée fut séchée sur sulfate de sodium anhydre et l'éther évaporé dans le vide, laissant une huile qui réfrigérée à —10°, cristallise. Recristallisé dans un mélange d'éther et d'éther de pétrole on a obtenu 2.5 g avec F 34—38°. Recristallisé encore 3 fois dans la ligroine (Éb. 60—100°) F est élevé jusqu'à 43—45°, rendement 0.95 g. Trouvé E (titration avec la soude) 144; C 49.88; H 5.50. Calc. ($C_6H_8O_4$): E 144.1; C 50.01; H 5.60.

Anschütz [17] a par réaction entre l'anhydride citraconique et le méthanol obtenu une huile qui se décompose par distillation dans le vide. Il n'indique pas l'analyse de la substance.

L'anhydride citraconique n'étant pas symétrique, deux esters-acides peuvent exister. Il faut donc se demander lequel des deux monoesters isomères a été isolé par le procédé décrit.

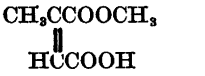
Pour l'acide méthylsuccinique Hancock et Linstead [28] ont vérifié la structure des deux monoesters méthyliques. Nous avons donc transformé l'ester-acide citraconique en l'ester correspondant saturé, c'est à dire le méthylsuccinate acide de méthyle, pour le comparer avec des spécimens authentiques des deux méthylsuccinates acides de méthyle.

A une solution de 0.5 g de citraconate acide de méthyle (F 43—45°) dans 20 ml de méthanol fut ajoutée une petite quantité d'oxyde de palladium, et la solution fut hydrogénée dans une atmosphère d'hydrogène pendant 24 heures (pression atmosphérique). Le méthanol fut enlevé par évaporation et le résidu recristallisé dans la ligroine. Rendement 0.37 g avec F 39—41°. Après encore deux recristallisations dans un mélange d'éther et de ligroine le rendement fut 0.2 g avec F 42—44°. Trouvé: C 49.48; H 6.83. Calc. ($C_6H_{10}O_4$): C 49.33; H 6.90.

Hancock et Linstead [28] indiquent les points de fusion suivants:

I. F 42.5—43.4 II F 23—24.2

Notre substance ne donne aucune dépression de point de fusion en la mélangeant avec I. Il faut donc en conclure que le citraconate acide de méthyle avec F 43—45° a la structure



et que cet ester acide est le produit principal de la réaction entre l'anhydride citraconique et le méthylate de potassium.

b. Citraconate acide d'éthyle. Le sel de potassium fut préparé sur le modèle du sel de l'ester-acide homologue inférieur. Partant de 5.6 g (0.05 mol) d'anhydride citraconique le rendement fut 8.5 g ou 86 %. E (titration avec l'acide perchlorique) 196; calc. 196.3.

Après deux recristallisations dans un mélange d'acétone et d'éthanol absolu on trouve C 42.58; H 4.54. Calc. ($C_7H_9O_4K$): C 42.85; H 4.63.

L'ester-acide fut isolé et obtenu comme une huile qui ne cristallise pas à $-10°$. Anschütz [17] à préparé l'ester-acide en traitant l'anhydride citraconique avec l'éthanol. Il le décrit comme une huile sans en donner l'analyse.

c. Citraconate acide de benzyle. Le sel de potassium fut préparé en ajoutant une solution de 1.95 g (0.05 atome) de potassium dans 35 ml d'alcool benzylique à une solution de 5.6 g (0.05 mol) d'anhydride citraconique dans 35 ml d'alcool benzylique. Après un repos d'une heure un litre d'éther fut ajouté. Le mélange fut placé dans la glacière jusqu'au lendemain et filtré à la trompe. Les cristaux furent lavés avec l'éther et séchés à l'air. Rendement 3.7 g. Une seconde précipitation (4.3 g) fut obtenue en ajoutant encore un litre d'éther au filtrat. Rendement total 8.0 g ou 70 %. E (titration avec l'acide perchlorique) 258; calc. 258.3.

L'ester-acide fut libéré en ajoutant l'acide chlorhydrique dilué à une solution de 2 g du sel de potassium dans 10 ml d'eau. La solution aqueuse fut extraite par 3 × 25 ml d'éther, la solution éthérée séchée sur sulfate de sodium anhydre et le solvant enlevé dans le vide. Le résidu huileux cristallise en le séchant dans le vide sur acide sulfurique concentré. L'ester-acide fut recristallisé dans un mélange d'éther et d'éther de pétrole. Rendement 1.2 g ou 77 %. F 78—80°. Recristallisé encore 3 fois dans le même mélange de solvants, F monte jusqu'à 99—102°. E (titration avec la soude) 219; C 65.50; H 5.35. Calc. ($C_{12}H_{12}O_4$): E 220.2; C 65.45; H 5.50.

Anschütz [17] a préparé l'ester-acide en traitant l'anhydride citraconique avec l'alcool benzylique. Il indique F 86°.

Étant donné que deux esters-acides peuvent résulter de la réaction entre l'anhydride et l'alcool ou son sel de potassium, la différence entre le point de fusion trouvé par nous et celui indiqué par Anschütz semble indiquer, que la proportion entre les deux isomères peut varier avec les méthodes de préparation aussi bien qu'avec le nombre des recristallisations. Nous ne savons pas si la préparation avec F 99—102° est encore un mélange des deux isomères ou une substance pure, et, le cas échéant, si la fonction carbonyle libre est celle la plus proche du groupement méthylique ou celle la plus éloignée de celui-ci.

Acide chloromaléique

a. Chloromaléate acide de méthyle. Le sel de potassium fut préparé en ajoutant lentement, sous agitation à la main, une solution de 3.9 g (0.1 atome) de potassium dans 50 ml de méthanol anhydre à une solution de 13.2 g (0.1 mol) d'anhydride chloromaléique dans 50 ml de méthanol anhydre, le mélange s'échauffant un peu pendant l'addition. Après un repos d'une heure, le sel de potassium fut précipité par addition de 1 500 ml d'éther, la précipitation étant complétée en laissant le mélange dans la glacière jusqu'au

lendemain. Le sel, une poudre blanche, fut isolé à la trompe, lavé avec l'éther et séché dans le vide sur acide sulfurique. Rendement 19.7 g ou 97 %. E (titration avec l'acide perchlorique) 203; calc. 202.6.

Le sel fut recristallisé deux fois dans un mélange de méthanol et d'acétone, prenant pendant la recristallisation 1/2 mol d'eau. On trouve après la recristallisation E (titration avec l'acide perchlorique) 210; C 28.33; H 2.63. Calc. ($C_5H_4O_4ClK$, $1/2H_2O$): E 211.6; C 28.35; H 2.38. En éstimant le chlore d'après Stepanov-Bacon on trouve E 210.

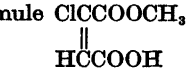
L'eau de cristallisation est enlevée en séchant la substance dans le vide sur anhydride phosphorique à 78°.

Pour obtenir l'ester-acide libre 7 g du sel de potassium furent dissout dans 20 ml d'eau, et la solution fut acidifiée avec l'acide chlorhydrique dilué. La solution acide fut extraite par 3 × 25 ml d'éther, la solution éthérée séchée sur sulfate de magnesium anhydre et l'éther enlevé dans le vide, laissant une huile qui par réfrigération à −10° cristallise. Après recristallisation dans un mélange d'éther et d'éther de pétrole le rendement fut 2.2 g ou 40 %. F 38−40°.

Recristallisé encore deux fois dans le même mélange de solvants F peut être élevé jusqu'à 42−44°.

E (titration avec la soude) 165.5; C 36.26; H 3.29; Cl 21.66. Calc. ($C_5H_5O_4Cl$): E 164.6; C 36.49; H 3.04; Cl 21.54.

Par titration potentiométrique dans 30 % éthanol on trouve pour pKs de l'ester monométhylique la valeur 3.19, pK_{s_1} de l'acide chloromaléique étant 1.72, pK_{s_2} 3.86 [20]. Nous pensons donc que l'ester a la formule



Par distillation dans le vide (1 mm de Hg) l'ester acide est transformé en anhydride chloromaléique.

b. Chloromaléate acide d'éthyle. Préparé par un procédé analoque à celui décrit pour l'homologue inférieur le sel de potassium du chloromaléate acide d'éthyle fut isolé avec un rendement de 92 %. Pour le produit brut on trouve E (titration avec l'acide perchlorique) 213; calc. ($C_6H_6O_4ClK$) 216.7.

Recristallisé deux fois dans un mélange d'éthanol et d'acétone le sel prend 1/2 mol d'eau. E (titration avec l'acide perchlorique) 225; C 31.76; H 3.10; Cl 15.62. Calc. ($C_6H_6O_4ClK$, $1/2H_2O$): E 225.7; C 31.93; H 3.15; Cl 15.72.

L'ester-acide fut libéré et isolé comme d'habitude, formant une huile qui ne cristallise pas à −10°.

c. Chloromaléate acide de benzyle. Le sel de potassium fut préparé en ajoutant pendant 15 minutes une solution de 3.9 g (0.1 atome) de potassium dans 100 ml d'alcool benzylique à une solution de 13.2 g (0.1 mol) d'anhydride chloromaléique dans 50 ml d'alcool benzylique, agitant le mélange à la main pendant l'addition. Après un repos d'une heure on ajoute 1 000 ml d'éther, et le mélange est laissé à la glacière à −10° jusqu'au lendemain pour compléter la précipitation du sel, qui est alors isolé à la trompe, lavé avec l'éther et séché dans le vide sur acide sulfurique. Rendement 26 g ou 93 %. E (titration avec l'acide perchlorique) 277; calc. ($C_{11}H_8O_4ClK$) 278.7.

Pour isoler l'ester-acide 10 g du sel de potassium furent recouverts de 150 ml d'eau (le sel n'étant pas complètement soluble dans cette quantité d'eau). En acidifiant la solution avec l'acide chlorhydrique dilué, l'ester-acide précipite huileux, et laissé dans la glacière, il cristallise. Rendement 7.2 g ou 84 %.

Recristallisé dans un mélange d'éther et d'éther de pétrole on ne retrouve que 3.8 g ou 44 % d'une substance avec F 60−65° et E (titration avec la soude) 240; calc. ($C_{11}H_9O_4Cl$) 240.6. Recristallisé deux fois dans la ligroine F s'elève à 69−70°. Trouvé C 54.50; H 3.83. Calc. C 54.90; H 3.77.

Le rendement faible de la recristallisation est dû au fait que l'huile précipitée par addition de l'acide chlorhydrique à la solution aqueuse du sel de potassium contient une partie qui est insoluble dans l'éther et qui consiste sans doute en sel acide de l'ester-acide, composé d'une molécule du sel de potassium et d'une molécule de l'ester-acide. La partie insoluble dans l'éther ne fut pas purifiée, mais par titration avec l'acide perchlorique on trouve E 540, par titration avec la soude E 485, tandis que la valeur calculée pour le sel double est E 519.3, c'est à dire que la partie insoluble dans l'éther contient un peu plus de l'ester-acide que du sel.

Traité de nouveau par l'acide chlorhydrique le sel double est transformé en ester-acide avec F 69—70°.

On a cherché à isoler deux esters monobenzyliques isomères de l'acide chloromaléique par précipitation fractionnée d'une solution aqueuse du sel de potassium, mais seulement la substance F 69—70° fut isolée. Il semble donc que l'un des esters isomères est formé exclusivement ou presque exclusivement.

Par titration potentiométrique dans 30 % éthanol on trouve pour pK_S de l'ester monobenzylique la valeur 3.35, c'est à dire que l'ester a la formule $ClC(COOCH_2C_6H_5)=CHCOOH$ (*cf.* p. 1682).

d. La préparation des sels de potassium du *chloromaléate mono-isopropylique* et *monocyclohexylique* fut essayée, mais sans résultat.

Acide phénylsuccinique

a. Phénylsuccinate acide de méthyle. A une solution de 4.4 g (0.025 mol) d'anhydride phénylsuccinique dans 20 ml de méthanol on ajoute une solution de 1 g (0.025 atome) de potassium dans 20 ml de méthanol. Après un repos d'une heure à la température ambiante le méthanol fut enlevé dans le vide, le résidu fut dissous dans 20 ml d'eau, la solution acidifiée avec l'acide chlorhydrique dilué et extraite par l'éther. La solution éthérée fut séchée sur sulfate de sodium anhydre, le solvant enlevé dans le vide et le résidu séché dans le vide sur acide sulfurique. Rendement 4.3 g ou 83 % d'une substance F 65—67° et E (titration avec la soude) 206; calculé ($C_{11}H_{12}O_4$) 208.2.

Anschütz [30] a préparé les deux esters monométhyliques isomères et en a prouvé la constitution. Il indique pour les deux substances F 92° et F 102°, de sorte que la substance avec F 65—67° obtenue par nous doit être un mélange des deux isomères. On a essayé de séparer les isomères par précipitation fractionnée d'une solution aqueuse des sels de potassium ou par recristallisation des esters dans un mélange d'éther et d'éther de pétrole, mais sans aboutir à une séparation complète.

Wegscheider et Hecht [11] ont obtenu le phénylsuccinate acide de méthyle (un mélange des deux isomères?) en traitant une solution benzènique de l'anhydride phénylsuccinique avec le méthylate de sodium, mais seulement avec un rendement faible.

b. Le phénylsuccinate acide d'éthyle fut préparé comme l'isomère homologue inférieur. Rendement (de 4.4 g de l'anhydride phénylsuccinique) 4.1 g ou 75 %. F 70—71°. E (titration avec la soude) 222; calc. ($C_{12}H_{14}O_4$) 222.2.

Les deux monoesters éthyliques isomères ont été préparé par Ramart-Lucas et Papadakis [31], qui indiquent pour les deux substances F 88—89° et F 95—96°. La substance avec F 70—71° doit donc être un mélange des deux isomères.

c. Phénylsuccinate acide de benzyle. A une solution de 4.4 g (0.025 mol) d'anhydride phénylsuccinique dans 20 ml d'alcool benzylique fut ajoutée une solution de 1 g (0.025 atome) de potassium dans 20 ml d'alcool benzylique. Après un repos d'une heure la solution fut extraite par 3 × 40 ml d'eau, la solution aqueuse fut extraite une fois par l'éther (pour enlever des traces de l'alcool benzylique) et ensuite acidifiée avec l'acide chlorhydrique dilué et extraite par 3 × 30 ml d'éther. La solution éthérée fut séchée sur sulfate de sodium anhydre et le solvant enlevé dans le vide. Le résidu cristallin fut recristallisé dans un mélange d'éther et d'éther de pétrole. Rendement 4.5 g ou 62 %. F 65—82°. E (titration avec la soude) 292; calc. 284.3.

Après plusieurs recristallisations dans la ligroine (Eb. 60—100°) on a obtenu (de 1 g du produit brut) 0.3 g avec F 102—103°; C 71.60; H 5.67. Calc. ($C_{17}H_{16}O_4$): C 71.83; H 5.67.

La préparation fut répétée, mais cette fois la solution aqueuse fut soumise à une acidification fractionnée avec l'acide chlorhydrique 1 N. On a ainsi obtenu 8 fractions:

a.	0.68 g avec F 90—95°	*e*	0.72 g avec F 80—85°
b.	0.72 g » F 80—85°	*f.*	0.70 g » F 80—85°
c.	0.72 g » F 80—85°	*g.*	0.73 g » F 78—80°
d.	0.71 g » F 75—80°	*h.*	0.32 g » F 76—81°

Rendement total 5.3 g ou 73 %.

Fraction *a* recristallisée deux fois dans la ligroine a donné 0.45 g avec F 101—103°
Fraction *b* + *c* recristallisées 4 fois dans la ligroine ont donné 0.65 g avec F 102—103°

Fraction *e* + *f* recristallisées 4 fois dans la ligroine ont donné 0.55 g avec F 101—102°.
Fraction *g* + *h* n'ont après 3 recristallisations dans la ligroine donné aucun produit avec point de fusion défini.
Fraction *d* recristallisée deux fois dans la ligroine a donné 0.58 g avec F 79—81° qui reste constant après encore deux recristallisations. Trouvé C 71.75; H 5.78. Calc. C 71.83; H 5.67.

Cette substance est donc un phénylsuccinate acide de benzyle isomère à la substance avec F 102—103°, qui est le produit principal de l'estérification.

Les spectres ultraviolets des deux esters acides sont à peu près identiques.

On a déterminé les p*K*s des deux esters acides dans une solution aqueuse-éthanolique avec 30 % d'éthanol. Pour la substance avec F 102—103° on trouve p*K*s 5.13, pour celle avec F 79—81°; p*K*s 5.20. Si cette différence est réelle, on en déduira que les deux esters acides auront les structures suivantes:

F 102—103°; p*K*s 5.13 F 79—81°; p*K*s 5.20

p*K*s, de l'acide phénylsuccinique étant 3.79. L'estérification du groupement carboxyle le plus fort semble donc être partiellement génée (empêchement stérique?).

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Crystallization of Normal Fatty Acids

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Normal fatty acids with 13, 15, 17 and 12, 14, 16, 18, 20, 22, 24, and 26 carbon atoms have been crystallized from pentane, ethyl ether, ethyl alcohol, acetone, ethyl acetate, benzene, carbon disulfide, carbon tetrachloride, and chloroform. The solid phases obtained have been investigated from the polymorphic point of view using a X-ray powder method.

Normal fatty acids exhibit polymorphism. The structures of the different polymorphic forms have been described by Vand, Morley and Lomer [1] (the C-form of lauric acid) and the author [2–7] (the A′- and B′-forms of *n*-pentadecanoic acid, the B-form of stearic acid and the C′-form of *n*-hendecanoic acid). At present the A-form of lauric acid is being investigated. The D′-form of acids with an odd number of carbon atoms, said to exist by some authors [8,9], has never been found in investigations carried out here in Uppsala. Information about it is very scanty [8,9].

By varying the mode of crystallization and the temperature, the different polymorphic forms can be obtained either singly or two together. This behaviour has been studied many times using X-ray methods, the most recent being by E. Stenhagen and the author [10]. (For earlier results see Ref.[10]).

In this investigation normal fatty acids with 13, 15, 17 and 12, 14, 16, 18, 20, 22, 24 and 26 carbon atoms have been crystallized from the following solvents: pentane, ethyl ether, ethyl alcohol, acetone, ethyl acetate, benzene, carbon disulfide, carbon tetrachloride, and chloroform.

MATERIAL USED

The acids used are the same as those used by Stenhagen and the author [10] and their preparation is described in Refs. [10-12]. The melting points of the acids given in these papers agree very well with those given by Francis and Piper [13].

EXPERIMENTAL

The crystallizations from the different solvents were carried out at +19° C and −14° C in order to study the influence of the rate of crystallization on the result.

Table 1.

Number of carbon atoms in the fatty acid	Temperature °C	Solvent									
		n-C_5H_{12}	$(C_2H_5)_2O$	C_2H_5OH	$(CH_3)_2CO$	$CH_3COOC_2H_5$	C_6H_6	CS_2	CCl_4	$CHCl_3$
13	+19°	A′	A′	A′	A′	A′	A′	A′	A′	A′
	−14°	A′	A′	A′	A′	A′	—	A′	A′	A′
15	+19°	A′	A′	A′	A′ ⩾ B′	A′	A′	A′	A′	A′
	−14°	A′	A′	A′	A′	A′	—	A′	A′	A′
17	+19°	B′	B′	B′	B′	B′	B′	B′	B′	B′
	−14°	B′	B′ > A′, A′ > B′, A′	B′	B′	B′ > A′	—	A′ ⩾ B′	B′ > A′	B′ > A′, A′

⩾ more of the first phase or equal.

Table 2.

Number of carbon atoms in the fatty acid	Temperature °C	Solvent								
		n-C_5H_{12}	$(C_2H_5)_2O$	C_2H_5OH	$(CH_3)_2CO$	$CH_3COOC_2H_5$	C_6H_6	CS_2	CCl_4	$CHCl_3$
12	+19°	C	C	C	C	C	C ≫ A	A	C ≫ A	A ≥ C
	−14°	A	A ≫ C	A ≥ C, A	A	A ≥ C	—	A	C ≥ A, A ≫ C	A ≥ C
14	+19°	C, A	C, A ≥ C	C	A ≥ C	C	C	A	C	C ≥ A
	−14°	A	A > C, C > A	C, A ≫ C	A ≫ C	A	—	A ≥ C	A	A
16	+19°	C	C ≥ A, A	C ≥ A	C ≥ A	C, A ≥ C	C ≫ A	C ≥ A	C ≫ A	C ≫ A
	−14°	A ≥ C	B	B	B	B	—	A ≥ B	C	A
18	+19°	C ≥ A	C, C ≥ B	C	C	C ≥ B	C ≥ B	C ≫ B	A ≥ C	C > B, B > C, B
	−14°	A ≥ C	C ≥ B	B	C	B	—	A ≥ C	A ≥ C	C ≥ B
20	+19°	C	C ≫ B, B	B ≥ C	C, C ≥ B	C ≥ B	C ≥ B	C ≥ B	C ≫ B, C ≥ B	B ≥ C
	−14°	C	B ≥ C	C ≥ B	C	C ≥ B	—	A ≥ C	A ≥ C	C ≥ B
22	+19°	C	C	C	C	C	C	C	C ≥ B	C ≥ B
	−14°	C	C ≥ B	C	C	C	—	C ≫ B	C	C
24	+19°	C	C	C	C	C	C	C	C	C
	−14°	C	C	C	C	C	—	C	C	C
26	+19°	C	C	C	C	C	C	C	C	C
	−14°	C	C	C	C	C	—	C	C	C

≥ more of the first phase or equal, ≫ much more of the first phase

In some cases samples were taken from two or three different parts of the crystallization vessel. The specimens were cut with a razor to a fine powder, which was cautiously pressed on Scotch tape fixed on the sample holder.

Powder photographs were taken in a Guinier camera using Cu*Ka* radiation.

The phase analysis was carried out partly by measuring the long spacing [10], *d*(001), partly by comparing the photographs with those obtained in connection with the crystal structure investigations [2-7].

RESULTS

The results are collected in Tables 1 and 2. The notations in each square belong to the same crystallization, but in some cases the crystalline solid has been divided into two or three parts before the phase analysis. In such cases the notations are given in order of decreasing rate of crystallization. *E. g.*, C>B, B>C, B means that the solid which crystallized first (on the walls of the crystallization vessel) consisted of more C- than B-phase. The next sample taken from the raised center of the bottom of the vessel consisted of more B- than C-phase, and in the periphery of the bottom, where the rate of crystallization was slowest, pure B-form was obtained.

The results will be discussed in a subsequent paper.

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On the Possible Interference of Electrode Reaction Products in Electrophoresis

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The relative merits of gassing and non-gassing electrodes for electrophoresis have been discussed in detail. Special attention has been paid to the buffer volume required between the electrodes and the separation zone. It has been found that platinum electrodes possess considerable advantages in spite of their gas evolution and that their use in electrophoresis instruments can be recommended, at least if not precise mobility measurements are concerned. It has been shown by theoretical reasoning and by conclusive experiments that the pH boundaries formed at such electrodes migrate towards the separation zone at quite moderate rates which have little to do with the excessively high mobilities of free H^+ and OH^- ions. The boundaries have been found to be very sharp, and the conditions for gravitational stability have been studied experimentally for some commonly used buffers. For ordinary analytical electrophoresis runs, a buffer volume 10 times the volume of the electrophoresis channel has been found to be sufficient. For prolonged runs, this volume can be retained if an acid lock is provided in the cathode vessel and an alkali lock in the anode vessel. The influence of these results on the construction of electrode vessels for an electrophoresis apparatus has been discussed.

The earliest workers in U-tube electrophoresis used platinum electrodes at the top of the limbs not far from the moving boundaries under observation (see *e. g.* Hardy[1] and Burton[2]). This caused the electrode reaction products to interfere with the substances under investigation.

It was a great advance when Michaelis[3] introduced special electrode vessels and an appreciable volume of electrolyte solution between the electrodes and the separation zone. He also introduced reversible electrodes. Svedberg and Jette[4] were still more cautious and used additional safety tubes between the electrode vessels and the U-tube. Tiselius[5] gave an equation for the buffer volume required between the electrodes and the U-tube in a run under specified conditions and of specified duration. Later[6], he pointed out that this volume could be reduced by using an increased buffer salt concentration in the electrode tubes, compared to that in the U-tube.

Electrophoresis instruments are now manufactured on a commercial basis in many countries. It is then worth while to reconsider the advantages and disadvantages of different types of electrodes, and to try to get conclusive evidence regarding the necessary buffer volume. A development in this direction has already been initiated by Antweiler [7], who revived the use of simple, irreversible electrodes. He also radically reduced the buffer volume, compared with earlier designs of electrophoresis cells. In paper electrophoresis instruments, which are also produced on a large scale, all constructors seem to have chosen platinum, or even simpler, irreversible electrodes, although the possible interference from electrode reaction products is the same as in the moving boundary method.

RELATIVE MERITS OF DIFFERENT TYPES OF ELECTRODES.

We will distinguish here mainly between gassing and non-gassing electrodes, since this is the most important property. A non-gassing electrode — generally silver-silver chloride electrodes are used — make it possible to use closed electrode vessels, which is regarded as very favourable for making accurate mobility measurements. Such electrodes are, however, not quite safe against gas evolution, unless they are handled very carefully and managed according to a strictly rigid running scheme (*cf.* Longsworth and MacInnes [8]). Such an electrode must have an appreciable area accessible to the solution in order to keep the risk for gas evolution as small as possible. To achieve that, comparatively much electrode material and a great deal of mechanical workmanship are required. The large dimension of the electrode and the desirability of having closed electrode vessels lead to the use of large and expensive ground joints. The cost of such an electrode together with an appropriate electrode tube must therefore be appreciable, and yet it cannot be managed by technical laboratory personnel without extensive training. In the most accurate mobility measurements a correction for volume changes at the electrodes is required even for non-gassing electrodes.

At a gassing electrode, an uncontrollable amount of gas bubbles sticks to the electrodes and to the adjacent glass walls. These bubbles, as they form and escape to the surface of the liquid, must be expected to give rise to slight back and forth movements of the boundary system and to a corresponding uncertainty in the localization of boundaries in the U-tube in free electrophoresis. In paper electrophoresis, on the other hand, they should be quite harmless. It is, however, always possible to remove such gas bubbles every time a measurement or an exposure is to be taken. Now, most investigators have only a minor interest in the measurement of mobilities and need only rough such values. Although the possibility of making mobility measurements between gassing electrodes has not been proven, the last-mentioned fact of course reduces the possible disadvantage of gas bubble formation.

Gassing electrodes are also connected with radical pH changes — in general, free acid is formed at the anode, and free alkali at the cathode — and with appreciable oxidation-reduction potentials. The former property has always been regarded as their least desirable property, partly because most substances investigated by electrophoresis are very sensitive to pH changes, partly since

the acid and alkali formed at the electrodes are believed, due to extremely high mobilities of the H^+ and OH^- ions, to reach the separation zone much more rapidly than concentration changes occurring at other electrodes. It will be shown later in this article that this argument does not apply in buffered media.

A very favourable property of a gassing electrode is the fact that it can be immersed directly into the electrolytic medium used in the run, whereas a non-gassing one requires a quite special electrolytic surrounding in order to function properly. The latter property necessitates a more complicated, and more expensive, electrode vessel. Further, a gassing electrode need not have a large surface; a short platinum wire is in general sufficient.

With due consideration to all the above points of view, it is quite evident that the difference in cost between the two types of electrodes, together with a properly constructed electrode vessel, is quite appreciable, even if the gassing electrode is made of platinum.

MOVING BOUNDAY SYSTEMS ORIGINATING AT NON-GASSING ELECTRODES.

Tiselius [5] gave the following equation for the volume V of buffer that has to be interposed between the U-tube and the electrolyte solution surrounding the electrode:

$$V = \frac{u\ v}{u_A - u_B}$$

where u_A and u_B are the mobilities of the components to be separated, v the desired volume of the separated components, and u the highest mobility of the ions in the solution round the electrodes. This equation is based on the assumptions that there is no convective transport of ions, and that the foreign ions travel towards the separation zone under the influence of the field strength prevailing in the buffer. In the light of Dole's [9] theory of moving boundary systems, we know now that none of these assumptions is necessarily true. As a matter of fact, the original boundary in the electrode tube between the electrode solution and the buffer will split up into a moving boundary system under the influence of the current in quite the same way as does the starting boundary in the U-tube. Consequently a number of moving boundaries are formed in each electrode vessel, each interboundary layer having its own density, conductance, and field strength. The different densities determine whether the migration will be convection-free or not, while the different conductances determine the absolute velocities of the boundaries.

The nature of the moving boundary systems present in the electrode vessels in the case of silver-silver chloride electrodes has been described by Brattsten [10] in an investigation that in thoroughness and clearness leaves nothing to be desired. She found that the moving boundaries originating from sodium chloride/buffer starting boundaries were, for many commonly used buffers, gravitationally unstable and gave rise to convection as soon as they were formed.

MOVING BOUNDARY SYSTEMS ORIGINATING AT GASSING ELECTRODES.

This discussion will be restricted to electrode/electrolyte systems that give rise to hydrogen gas and alkali at the cathode and to oxygen gas and acid at the anode. The moving boundaries originating from such electrodes can thus be characterized as pH boundaries. Very little is known concerning the behaviour of such boundaries in buffered media. A direct application of eqn. (1), using the mobilities of free H^+ and OH^- ions, would in this case lead to excessively large buffer volumes, due to the extremely high values of these mobilities. There are, however, reasons to believe that acid and alkali boundaries migrate much more slowly in buffered media, because hydrogen and hydroxyl ions entering a buffering zone will be extensively converted into water or into other, charged or uncharged, particles. Some simple experiments which support this view will be reported in the next sections of this article, but a more extensive investigation, including a theoretical treatment of this topic, would be highly desirable.

The pH boundaries, too, may be gravitationally stable or unstable, and it is very important to establish the conditions for convection-free boundary migration and to construct the electrode vessels accordingly. The author has made some simple experiments in order to reveal the most important facts.

Two beakers and a glass tube bridge between them were filled with the buffer solution in question. A trace of an acidimetric indicator, showing colour changes at low as well as at high pH values was added to the buffer. One platinum electrode was put into each beaker, and a direct current was sent through for some time. At the top of the anode beaker, a liquid layer with the acid colour of the indicator was then formed, and at the bottom of the cathode beaker, a layer with the alkaline colour developed (Fig. 1). The result was the same with all different buffers tested: phosphate, mixed phosphate and chloride, acetate, and barbital buffers with sodium as the cation.

These results can of course not be generalized by saying that the electrolytically generated acid is always lighter and the alkali always heavier than the buffer. This has to be tested experimentally in each special case as long as no theoretical treatment is available. Any experiments with ammonium salts

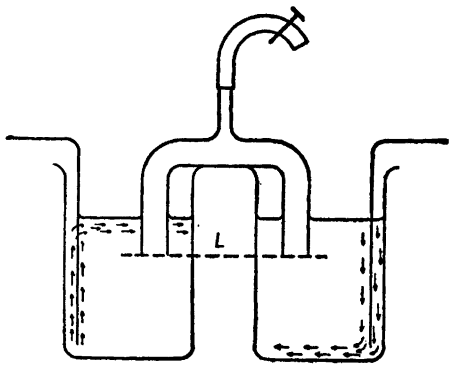

Fig. 1. Showing liquid streamings at platinum electrodes during electrolysis of common buffer solutions with sodium as cation. Electrolytically generated free acid, being lighter than the buffer, collects at the surface of the anode beaker, while the opposite holds for the generated free alkali in the cathode beaker. Acid enters the siphon when the buffer volume above level L has been replaced by acid. Alkali enters the siphon when the volume below level L has been replaced by alkali.

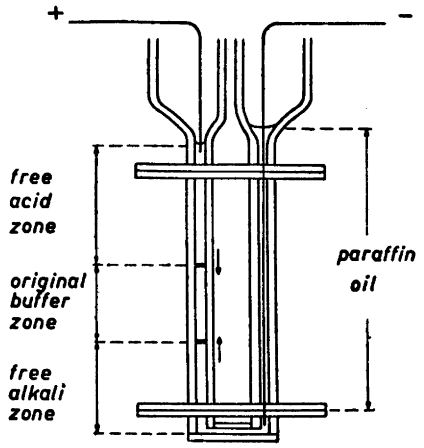

Fig. 2. Experimental arrangement for simultaneous measurement of migration rates of descending acid and ascending alkali boundaries.

have not been carried out, but it is quite certain that they would give the opposite result in the cathode compartment, since free ammonia is even lighter than water. The importance of these simple experiments, as well as of Brattsten's more extensive study, lies in the revealed fact that, in both cases, we have to do with convective ion transport, and that the problem of keeping foreign ions round the electrodes away from the separation zone is not simply a question of a minimum buffer volume in between. A convective ion transport may traverse any volume in a very short time. The bad effects of this convective transport, the ignorance of which has probably caused much trouble in accurate mobility measurements in many laboratories, can only be eliminated by an appropriate design of the electrode vessels. Such designs will be shown later in this article.

Another very interesting and important conclusion from the experiments mentioned above is the fact that the acid and alkali zones were found to grow with sharp fronts in the case of gravitational stability. This means that, with the buffer systems mentioned, an electrolytically generated zone can migrate with a stable, sharp, descending boundary, while an electrolytically generated alkali zone can migrate with a stable, sharp, ascending boundary. This is of course favourable, since sharp fronts do not require a great safety margin to be applied to calculated buffer volumes.

MIGRATION SPEEDS OF ACID AND ALKALI FRONTS IN BUFFERED MEDIA.

For such measurements, the experimental arrangement shown in Fig. 2 was used. In an electrophoresis U-tube according to Tiselius, the left-hand limb of the U-tube, the bottom section, and a small part of the left-hand part of the top section were filled with the buffer solution to be investigated. The right-hand limb of the U-tube and a small portion of the right-hand part of the top section were filled with paraffin oil. The platinum anode was inserted in the left-hand top compartment, while the cathode was put into the bottom sec-

tion, through the paraffin oil layer. After starting the direct current through the U-tube, one descending and one ascending boundary, both exceedingly sharp, could readily be seen in the camera. The migration rates of these boundaries were measured for some different, commonly used buffers.

In order to interpret these measurements, the following working hypothesis was put forward, with reference to Fig. 2. Experimentally it was found that the boundaries migrated at constant rates if the current was kept constant. Moreover, it was assumed that the three liquid phases, the acid phase at the top, the alkali phase at the bottom, and the buffer phase in between, all have constant compositions during the migration of the boundaries towards each other. If this hypothesis were true, both migration rates could be predicted since the buffer cationic radical disappears at the acid boundary, and the buffer anionic radical disappears at the alkali boundary. Consequently, if the field strength in the buffer phase is calculated from the equation:

$$E = \frac{i}{q\,\varkappa} \tag{2}$$

where $\varkappa$ is the conductance of the original buffer solution, q the cross section area, and i the current, and if the observed migration speeds are divided by this field strength, two mobilities would be obtained which would be expected to check with the mobilities of the two ion constituents of the buffer. Further, if these mobilities were multiplied with the respective equivalent concentrations, then added, and again multiplied with the Faraday constant, then the conductance of the original buffer solution would be obtained, according to the well-known formula:

$$\varkappa = F \sum z_i\, c_i\, u_i \tag{3}$$

where z_i are valences, with signs, c_i molar concentrations (positive), and u_i mobilities, with signs.

The working hypothesis was thus very easy to control experimentally. In Table 1, the necessary experimental data are collected, together with the conductivity check outlined above. The conclusion to be drawn from this table is that the working hypothesis does not hold quantitatively, since the calculated conductivities only have the correct order of magnitude. Nevertheless, the experiments show that the migration rates of the acid and the alkali fronts have little to do with the mobilities of free hydrogen and hydroxyl ions. They are instead approximately dictated by the mobilities of the ion constituents of the buffer.

The experiments were, for the sake of pure curiosity, continued after the two boundaries had met. It was then found that they united into one boundary (which could be expected) migrating downwards at a lower speed than both original boundaries. The explanation of this may lie in the following mechanism. If, still, the working hypothesis were true, the lower, purely alkaline phase would border directly on the upper, purely acidic phase, and the current would tend to pull the alkali solution further down, to pull the acid solution further up, and to develop a layer of pure water in between. Such a

Table 1. Showing four experimental tests of the possibility of calculating the conductivity of the buffer from the migration rates of the acid and alkali fronts.

Measured and calculated conductances ohm^{-1} cm^{-1}	Ion constituent	Concentration c cm^{-3}	Valence z	Mobility u $cm^2/voltsec$	Product czu
$2.66 \cdot 10^{-3}$	Na^+	$68 \cdot 10^{-6}$	+1	$+24.5 \cdot 10^{-5}$	$+1\,666 \cdot 10^{-11}$
	H^+	$40 \cdot 10^{-6}$	+1	$-42.9 \cdot 10^{-5}$	$-1\,716 \cdot 10^{-11}$
	PO_4^{---}	$36 \cdot 10^{-6}$	−3	$-42.9 \cdot 10^{-5}$	$+4\,633 \cdot 10^{-11}$
$4.42 \cdot 10^{-3}$	←	× 96 496	←		$+4\,583 \cdot 10^{-11}$
$2.35 \cdot 10^{-3}$	Na^+	$1 \cdot 10^{-4}$	+1	$+18.2 \cdot 10^{-5}$	$+18.2 \cdot 10^{-9}$
	H^+	$1 \cdot 10^{-4}$	+1	0	0
	Ac^-	$2 \cdot 10^{-4}$	−1	$-7.03 \cdot 10^{-5}$	$+14.06 \cdot 10^{-9}$
$3.11 \cdot 10^{-3}$	←	× 96 496	←		$+32.26 \cdot 10^{-9}$
$3.73 \cdot 10^{-3}$	Na^+	$1 \cdot 10^{-4}$	+1	$+19.7 \cdot 10^{-5}$	$+19.\ 7 \cdot 10^{-9}$
	H^+	$3 \cdot 10^{-4}$	+1	0	0
	Ac^-	$4 \cdot 10^{-4}$	−1	$-6.3 \cdot 10^{-5}$	$+25.\ 2 \cdot 10^{-9}$
$4.33 \cdot 10^{-3}$	←	× 96 496	←		$+44.9 \cdot 10^{-9}$
$3.78 \cdot 10^{-3}$	Na^+	$1 \cdot 10^{-4}$	+1	$+19.9 \cdot 10^{-5}$	$+19.9 \cdot 10^{-9}$
	H^+	$3 \cdot 10^{-4}$	+1	0	0
	Ac^-	$4 \cdot 10^{-4}$	−1	$-5.9 \cdot 10^{-5}$	$+23.6 \cdot 10^{-9}$
$4.20 \cdot 10^{-3}$	←	× 96 496	←		$+43.5 \cdot 10^{-9}$

(In the phosphate experiment, the mobility of the hydrogen ion constituent must be assumed to be equal to that of the phosphate ion constituent, since all hydrogen is bound to the phosphate ions. In the acetate experiments, the mobility of hydrogen must be assumed to be zero, since practically all hydrogen is present in the undissociated species HAc.)

system is apparently gravitationally unstable and causes the acid to fall down again on top of the alkali layer. Although the current tends to concentrate the acid more and more at the anode, the density relationships and convective currents cause the acid solution to become more and more dilute, while in the bottom compartment the alkali can concentrate without interference from convection. The fact that the working hypothesis was found not to be true does not completely invalidate this theory; it is only necessary to modify it.

Next, it was tried to find out what was wrong with the working hypothesis. Since the calculated values of the buffer conductances were consistently higher than those measured, it could be suspected that the buffer ions actually did not disappear completely at the migrating sharp fronts, but were lagging behind. This could be shown to be the case in two ways. By using the newly developed, more sensitive interferometric techniques (Svensson [11,12]), the sharp fronts were actually found to possess a tail on the side towards the

electrode, indicating a non-constant composition just behind the front. In addition, experiments were performed in a preparative way as follows.

In our research model electrophoresis apparatus, the cathode vessel, the cathode limb of the U-tube, and the bottom section were filled with sodium hydroxide of a density well above that of the buffer. This consisted of a mixture of monochloroacetic acid and its sodium salt, and it was filled in the rest of the apparatus. On closing the current, a very sharp alkali front migrated upwards in the anode limb of the U-tube. Its migration rate was measured. In order to control, however, if chloroacetate ions were lagging behind this boundary, it was allowed to migrate through one whole U-tube cell section, which was then pushed to the side after breaking the current. The contents of said cell section were then tested for chloride ions with silver nitrate and nitric acid, after the chloroacetate had been completely hydrolyzed by strong sodium hydroxyde at 100° C.

It was found that chlorine was consistently present below the ascending alkali boundary. This explains why the working hypothesis presented above could not give quantitative agreement with experiments.

It was also found that the mobility, calculated from the migration velocity of the ascending alkali boundary and the field strength in the buffer layer, varied in a regular manner with the proportion between chloroacetate and free chloroacetic acid. This is shown in the graph in Fig. 3. The mobility of the alkali boundary rises very rapidly as the concentration of excess acid decreases, *i. e.* as the buffering capacity decreases. As a matter of fact, if one tries to

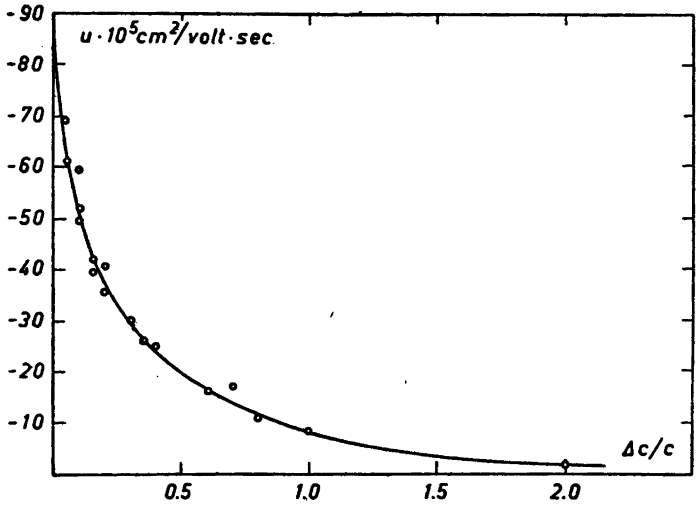

Fig. 3. Migration velocity of alkali front moving into a sodium chloroacetate buffer, per unit field strength in the buffer, as a function of the relative excess of free acid in the buffer, at an ionic strength of 0.1 and a temperature of 0 °C. The value extrapolated to zero acid excess checks with the mobility of free hydroxyl ions (u_{OH} at infinite dilution $= -109 \cdot 10^{-5}$), but already at a relative excess of 0.5, the alkali front migrates more slowly than the buffer anion ($u_{ClAc} = -18.5 \cdot 10^{-8}$).

extrapolate this mobility to zero acid excess, one gets a figure not far from the mobility of free hydroxyl ions. The extrapolation is difficult, however, since the very sharp front characterizing the alkaline zone gradually becomes more diffuse as the excess free acid is decreased.

CONSEQUENCES AS TO THE CONSTRUCTION OF ELECTRODE VESSELS.

The most important result of the above discussion and experiments is the fact that one always has to take into account the density relationships of the moving boundary systems developed by the current in the electrode vessels. Some moving boundaries are gravitationally stable on migration upwards and will give rise to convection if they are forced to migrate downwards, and *vice versa*. An electrode vessel must therefore necessarily be constructed so that the current has a vertical, downward direction in one part of it, while in another part it has an upward direction. By courtesy of Dr. Brattsten, the author was informed of her experimental results at an early stage, which made it possible to incorporate the electrode vessel shown in Fig. 4 into our research model electrophoresis apparatus types LKB 3021 and LKB 3023, which were developed in the years 1948/49. In the central part of this tube, the current flows downwards, while in the outer mantle, it flows upwards, or *vice versa*. A moving boundary gravitationally unstable in one part, is stable in the other. In any case, therefore, there is an appreciable volume of buffer that is effective in delaying the interference of electrode reaction products with substances in the separation zone.

In the paper electrophoresis apparatus described by Valmet and Svensson [13], the electrode vessels are constructed in a similar manner.

ACID AND ALKALI LOCKS.

The experiments reported here have shown that the buffer volume required between electrodes and separation zone is not greater for platinum than for silver-silver chloride electrodes. A volume 10 times as large as that of the separation zone seems to be sufficient under standard experimental conditions. This volume is so small, that there is no need for reducing it any further. If, however, an apparatus constructed for standard conditions has to be used in a prolonged electrophoretic separation, the capacity of the standard electrode vessels is of course insufficient, and the question arises how to circumvent this obstacle without making bigger vessels. Of course it is possible to make use of Tiselius' suggestion of having an increased salt concentration in the electrode vessels, as referred to in the introduction, but, in the case of gassing electrodes generating acid and alkali, there is a much more effective way of delaying the advance of the pH boundaries coming from the electrodes. This consists in the use of acid and alkali locks and has already been described by the author [14] in another connection. Since that publication is not easily available everywhere, however, the principle will be presented here again.

Let us suppose that we are using an acetate buffer, and that we want to delay the advancing alkali front in the cathode tube, which may be assumed to

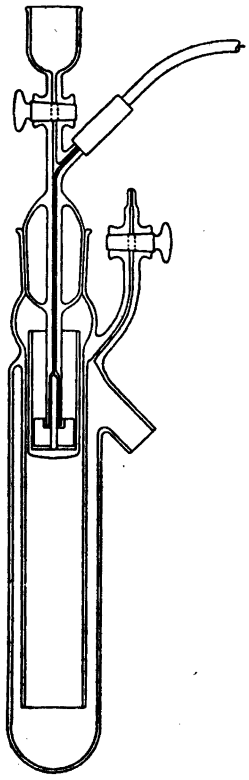

Fig. 4. Electrode vessel belonging to LKB 3021 and 3023 research model electrophoresis apparatus. The volume is subdivided into two parts, which forces the current to pass upwards in one part and downwards in the other. A moving boundary which is gravitationally unstable in one part can always migrate convection-free in the other part.

have the construction according to Fig. 4, but with a platinum electrode instead of the silver one shown in the figure. That can be done by placing a suitable volume of strong acetic acid in the bottom of the tube. This solution must of course be heavier than the buffer. The alkali generated at the electrode will then have to neutralize this acid before it can start to develop an ascending boundary in the outer mantle of the tube. The necessary quantity of acid can easily be calculated by a simple application of Faraday's electrolytic laws. If the acid in question is slightly soluble (barbital), a paste of crystals can be used instead of a concentrated solution.

The new starting boundary between acetic acid and acetate buffer that is introduced in this way will of course develop a moving boundary system during the electrolysis. No boundary migrating towards the U-tube can, however, be expected, since there is only one negative ion constituent in the system (*cf.* Dole's theory). The only moving boundary developed from this starting boundary is a pH boundary migrating away from the separation zone.

In the anode vessel, the acid boundary can be correspondingly delayed by an alkali lock. In the case of ammonia or other light bases, it must be remembered that the required density must be achieved by the addition of a suitable non-electrolyte, *e. g.* sugar.

The principle of acid and alkali locks should not be applied in mixed buffers. As an example, let us suppose that we are using a phosphate-chloride buffer and that a mixture of phosphoric and hydrochloric acids is used as an acid lock in the bottom of the electrode tube in Fig. 4. According to Dole's theory, this starting boundary will give rise to one boundary moving in the direction towards the U-tube. This boundary may also be gravitationally unstable and thus give rise to convection. Only if a certain relation between the two acid concentrations is satisfied does this moving boundary disappear, but this relation is very difficult to predict without experiments.

The principle of acid and alkali locks has been used in this laboratory on many occasions and is, according to our experience, both simple to apply and effective.

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The Influence of Temperature on the Rate of Enzymic Processes

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Enzymes may be reversibly denatured at low temperatures. The enthalpy of activation ΔH^* (or Arrhenius' energy of activation μ), the enthalpy of denaturation ΔH, and the temperature for half denaturation $t_{\frac{1}{2}}$ can easily be computed from measurements of the activity at various temperatures. Earlier computations of the heat of activation for enzymic processes, founded on measurements done in a comparatively short interval of temperature, gave probably not the true value of the heat of activation but the sum of the true value and part of the negative value of the heat of denaturation. For the hydrolysis of tributyrin with pancreatic lipase at pH 8.1 $\Delta H^* = 4\,700$ cal/mole, $\Delta H = -27\,000$ cal/mole, and $t_{\frac{1}{2}} = 3$ °C. For the hydrolysis of saccharose with yeast saccharase at pH 4.45 $\Delta H^* = 10\,000$ cal/mole, $\Delta H = -51\,000$ cal/mole, and $t_{\frac{1}{2}} = -4$ °C. For the hydrolysis of casein with trypsin at pH 8.1 $\Delta H^* = 11\,000$ cal/mole, $\Delta H = -53\,000$ cal/mole, and $t_{\frac{1}{2}} = 0$ °C.

The influence of temperature on the rate of chemical processes was successively interpreted by van't Hoff [1] in 1884, by Arrhenius [2] in 1889 and by Eyring [3,4] in 1935.

It is usual to express this influence in one of the following two ways. It can be given as the energy of activation μ according to Arrhenius' equation which is generally written

$$k = PZe^{-\mu/RT} \tag{1}$$

or

$$\frac{\mathrm{d} \ln k}{\mathrm{d}\,(1/T)} = -\frac{\mu}{R} \tag{2}$$

where k is the rate constant, P is the steric factor [5], Z is the number of collisions, μ the energy of activation, T the absolute temperature and R the gas constant.

The influence of temperature can also be expressed as the enthalpy of activation ΔH^* according to Eyring's equation:

$$k' = \frac{\varkappa kT}{h}\, e^{\Delta S^*/R}\, e^{-\Delta H^*/RT} \tag{3}$$

where k' is the specific reaction rate, $\varkappa$ is the transmission constant, k is Bolzmann's constant, h is Planck's constant, ΔS^* is the entropy of activation, and ΔH^* is the enthalpy of activation. The enthalpy of activation ΔH^* is usually calculated as $\Delta H^* = \mu - RT$ (T is here the average of the temperatures at which the experiments for the determination were carried out), but it has recently been shown by the present author [6] that it can also be calculated according to the following equation:

$$\frac{\mathrm{d} \ln (k/T)}{\mathrm{d} (1/T)} = -\frac{\Delta H^*}{R} \tag{4}$$

by plotting the logarithms of the rate constants divided by the absolute temperatures *versus* the inverse values of the absolute temperatures, and fitting a straight line to the points; the enthalpy of activation is obtained as the negative value of the inclination of the line multiplied by the gas constant R.

It is thus rather simple to characterize the change of the reaction rate with temperature for most chemical reactions. However, in the case of enzymic reactions, if the logarithms of the reaction rates are plotted *versus* the inverse values of the temperatures, and if the temperature interval is not small, it has been observed that the points usually do not lie about a straight line. The results from such investigations have recently been reviewed by Johnson, Eyring and Polissar [7].

In most investigations concerning the heat of activation of enzymic reactions, the temperature interval has been so small that a deviation from a straight line could not be demonstrated with any certainty. Hence a straight line was fitted in the corresponding Arrhenius plot. The results thus obtained are, of course, valid only as apparent heats of activation for the interval in question. For a list of such results the reader is referred to the reviews by Eyring and Stearn [8] and by Sizer [9].

Two cases of deviations from a straight line may be considered here. In one, best known from the investigations on luciferase, the reaction rate increases with the temperature at low temperatures, passes a maximum and then decreases reversibly as the temperature increases. In the other case, which is known from several enzyme systems, the reaction rate successively decreases with the temperature more than would be expected if the heat of activation at ordinarily high temperatures remained constant when the temperature was lowered. Several explanations have been given for this behaviour of enzymic reactions.

For the first case mentioned, exemplified by luciferase, Johnson *et al.*[7,10] and Eyring [11] gave the explanation that the enzyme is denatured as the temperature increases; however, this denaturation is not irreversible as in most other systems described but reversible with a definite equilibrium constant. A reversible denaturation at high temperatures is also known from investigations on other enzymes, *e. g.*, trypsin [12].

In the second case, as already mentioned, the rate successively decreases with decreased temperature more than would be expected if the heat of activation remained constants. For this case Sizer [13] suggested that two straight lines should be fitted in the Arrhenius plot and gave the explanation that the

sharp break in the temperature relationship indicates that the kinetics of the enzyme action is very different above and below this point. This suggestion is similar to the earlier suggestion of Blackman, Pütter and Crozier (see Ref. [7,14]) for the influence of temperature on the rate of biological processes: there is supposed to be a catenary series of chemical reactions with their own values for the heat of activation μ. Kistiakowsky and Lumry [15] upon a kinetic analysis concluded that the existence of sharp breaks is rather unlikely. In one case, in which the existence of a sharp break had been claimed — urease in the presence of sulfite ions — they found that the apparent activation energy rises gradually and not suddenly as the temperature is lowered, and that the change in the apparent activation energy can be explained by the temperature dependence of reversible inhibition of urease by sulfite ions. It may be mentioned here that as early as 1930 Buchanan and Fulmer [16], in discussing the influence of temperature on the rate of carbon dioxide production in alcoholic fermentation, drew attention to the wantonness of dividing a smooth curve into several pieces of straight lines.

Another explanation for the behaviour of enzyme systems at low temperatures has been suggested by Kavanau [17]: there may be reversible denaturation of an enzyme at low temperatures, comparable to the reversible denaturation of some enzymes at high temperatures. However, Kavanau, in his further treatment of this problem, immediately leaves this theory and gives a description of how to fit a complicated curve (including the incomplete gamma function) to the experimental results. For this curve-fitting, which has no clear theoretical significance [7], Kavanau makes use of three parameters, whereas Arrhenius' equation has only two (usually denoted PZ and μ), and hence Kavanau gets a curve which is close to the experimental points over a wide interval.

A THERMODYNAMIC INTERPRETATION

In this paper, the present author suggests a reversible inactivation of enzymes at low temperatures (first surmised by Kavanau [17]) as an important cause for the temperature dependence of many enzymic processes *.

Theory

Let E_a denote the active form of the enzyme, E_i the inactive form, and K the equilibrium constant, and let brackets denote concentrations. It is reasonable to consider here only the case that one molecule of this active form of the enzyme gives one molecule of the reversibly inactive form.

We thus get the following equations:

$$E_a \rightleftharpoons E_i \tag{5}$$

$$\frac{[E_i]}{[E_a]} = K \tag{6}$$

$$\frac{[E_a]}{[E_a] + [E_i]} = \frac{1}{1 + K} \tag{7}$$

* After this paper was prepared, a paper was published by Maier, Tappel, and Wolman [22], who use the same theory for peroxidase and phosphatase.

From thermodynamics the following relation between an equilibrium constant K, the absolute temperature T, the gas constant R and the enthalpy ΔH is well-known:

$$\frac{\mathrm{d} \ln K}{\mathrm{d}\, 1/T} = -\frac{\Delta H}{R} \tag{8}$$

If half of the total amount of enzyme is in the active form at the temperature $T_{\frac{1}{2}}$, which implies that the equilibrium constant has the value 1, then we get on integration of eqn. (8).

$$\ln K = -\frac{\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_{\frac{1}{2}}}\right) \tag{9}$$

and

$$K = \exp\left[-\frac{\Delta H}{R}\left(\frac{1}{T} - \frac{1}{T_{\frac{1}{2}}}\right)\right] \tag{10}$$

Now having considered the reversible inactivation of the enzyme, we shall turn to the enzymatically catalyzed reaction. For the influence of temperature on the rate of this reaction we have Arrhenius' and Eyring's equations, already given here as eqns. (1) and (3) which in this form are valid if all of the enzyme is active. If only part of the enzyme is active but the specific reaction rate is calculated for the total amount of enzyme, we get the following expression by combination of eqns. (1) and (7)

$$k = \frac{PZ}{1 + K}\, \mathrm{e}^{-\mu/RT} \tag{11}$$

and

$$k' = \frac{\varkappa kT}{h} \cdot \frac{\mathrm{e}^{\Delta S^*/R} \cdot \mathrm{e}^{-\Delta H^*/RT}}{1 + K} \tag{12}$$

This expression is mathematically equivalent to the expression which is valid for the influence of temperature on the rate of an enzymic reaction if there is a reversible denaturation of the enzyme at *high* temperatures (see *e. g.*, Ref. [7], eqn. (8, 4)), whereas the expression here is valid for the denaturation at low temperatures.

From eqns. (11) and (12) we get the following equations for the asymptote of the curve in an Arrhenius' plot or the plot suggested by the present author [6] by multiplying with $1 + K$, and by deriving logarithmically

$$\frac{\mathrm{d} \ln k\,(1 + K)}{\mathrm{d}\, 1/T} = -\frac{\mu}{R} \tag{13}$$

$$\frac{\mathrm{d} \ln k\,(1 + K)/T}{\mathrm{d}\, 1/T} = -\frac{\Delta H^*}{R} \tag{14}$$

The computation of ΔH^*, ΔH, and $T_{\frac{1}{2}}$

The computation of ΔH^*, ΔH, and $T_{\frac{1}{2}}$ can be done by successive approximations. The procedure will be exemplified with the data of Sizer and Josephson [13] for the hydrolysis of tributyrin with pancreatic lipase in the presence of 36 % glycerol.

Table 1. Example of the computation of the equilibrium constant K *for the low temperature denaturation of lipase from Sizer and Josephson's* [18] *measurements.*

1. The temperature for the experiments. 2. The time in hours for 1 % hydrolysis of tributyrin. 3. The abscissa values for Figs. 1—3. 4. The ordinata values of the experimental plots in Figs. 1 and 3. 5. The differences between the ordinata values for the asymptote and the plots as read in Fig. 1. 6. The ordinata values for Fig. 2.

1	2	3	4	5	6
°C	$1/k$	$10^4/T$	log k/T	log $(1+K)$	log K
50.0	0.0183	30.94	0.228 — 1		
40.0	0.0198	31.93	0.208 — 1		
30.0	0.0325	32.99	0.006 — 1		
20.0	0.0850	34.11	0.603 — 2		
10.0	0.0800	35.32	0.645 — 2		
5.0	0.133	35.95	0.432 — 2		
— 0.2	0.137	36.58	0.427 — 2		
— 4.5	0.550	37.22	0.830 — 3	0.774	0.693
— 4.5	0.400	37.22	0.969 — 3	0.635	0.521
— 5.0	0.50	37.29	0.873 — 3	0.724	0.633
— 9.0	1.62	37.86	0.369 — 3	1.171	1.140
—10.0	8.50	38.00	0.650 — 4	1.874	1.868
—12.0	4.0	38.29	0.981 — 4	1.514	1.500
—15.0	3.5	38.74	0.044 — 3	1.405	1.388
—18.0	7.0	39.19	0.748 — 4	1.655	1.645
—18.0	4.0	39.19	0.991 — 4	1.412	1.395
—18.0	7.5	39.19	0.718 — 4	1.685	1.676
—24.5	87.5	40.22	0.662 — 5	2.636	2.635
—24.5	55.0	40.22	0.864 — 5	2.435	2.433

The values of log k/T are plotted *versus* $1/T$ (if one wishes to get μ instead of ΔH^*, the values of log k are plotted *versus* $1/T$). As the temperature increases, the reversible denaturation at low temperatures decreases, and thus, in the diagram, the plots asymptotically approach a straight line. This asymptote is drawn by the eye (Fig. 1) as is the first estimation of ΔH^*. The differences between the corresponding ordinates for the asymptote and the points at low temperatures are read in the diagram; they give the values of log $(1 + K)$ (*cf.* eqn. (14)). The values of log K are calculated (Tab. 1) and plotted versus $1/T$ in another diagram (Fig. 2) (*cf.* eqn. (8)). A straight line is fitted to the points *, and the heat of denaturation ΔH is calculated from the inclina-

* It can be seen from Fig. 2 that one of the points lies remarkably distant from the line. If all points are considered for calculating the regression line, the standard deviation calculated [18] according to the formula $s_{y \cdot x}\sqrt{1 + 1/n + x^2/\Sigma x^2}$ at the abscissa of the points is 0.32, whereas the difference in ordinata for the point and the line is 0.714, which is $t = 2.22$ times the standard deviation. The probability for this distance in random sampling is 5 %. If the regression line is calculated without this point, the standard deviation at the abscissa of the point is 0.21 and the difference in ordinata for the point and the line is 0.80, which is $t = 3.7$ times the standard deviation. The probability for this distance in random sampling is between 1 and 0.1 %. We have thus statistical reasons for excluding this point from the calculations.

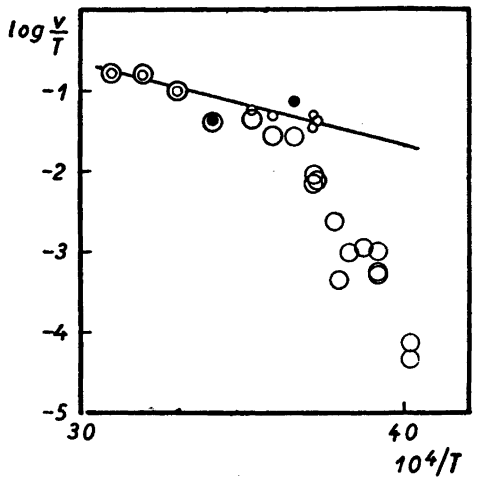

Fig. 1. Fitting an asymptote to a plot[6] of the activity of lipase at various temperatures as measured by Sizer and Josephson [13] for the computation of the enthalpy of activation ΔH and the equilibrium constant* K *between denatured and native lipase (*cf. *Table 1 and 2). Large rings: experimental values, log* k/T. *Small rings: experimental values corrected for reversible low temperature denaturation. Dots: experimental values which because of specified reasons were not regarded in fitting the line. In the first approximation, the straight line is fitted by the eye as the asymptote to the plots. In the second approximation, the straight line is fitted in the normal way to the corrected plots. Both log* k/T *and log* k(1 + K)/T *are thus plotted as ordinata values of log* V/T.

tion of the line in the usual way as the product of —1, the inclination, the gas constant $R = 1.9872$ cal · degree^{-1} · mole^{-1}, and ln 10 = 2.3026. In this diagram the half activity temperature $T_{\frac{1}{2}}$ is also read as the temperature, for which the logarithm of the equilibrium constant has the value 0.

The second approximation is thereafter done as follows. The values of ΔH and $T_{\frac{1}{2}}$ are used for the calculation of the values of log $(1 + K)$ for the relatively high experimental temperatures. The values of log K can be read in the diagram by extrapolation, and then log $(1 + K)$ and, finally, log $k(1 + K)/T$ are computed from these readings (Table 2). These values are now plotted versus $1/T$ (*cf.* eqn. (14)), and a straight line is fitted to the points *. (If one wishes to get μ instead of ΔH^*, the values of log $k(1 + K)$ are plotted in an Arrhenius diagram.) This line is a better estimation of the true asymptote than the line previously drawn by the eye. The inclination is read (or calculated [18] by the method of least squares) and the value of the enthalpy of activation ΔH^* is calculated as usual (eqn. (14)). If the value for the inclination of the asymptote obtained after this approximation differs appreciably from the first estimation, the whole calculation is repeated with the new value.

* In this fitting two points have been excluded because of the following reasons. The plot of the experiment at 10 °C (fourth plot fom the left) lies remarkably lower than in Sizer and Josephson's Fig. 1, and there is probably a misprint in their Table 1. The other point which was excluded, at —0.2 °C (the seventh point from the left) is remarkably distant from the line; a statistical calculation gives the value $t = 10$, which corresponds to a probability less than 0.1 %.

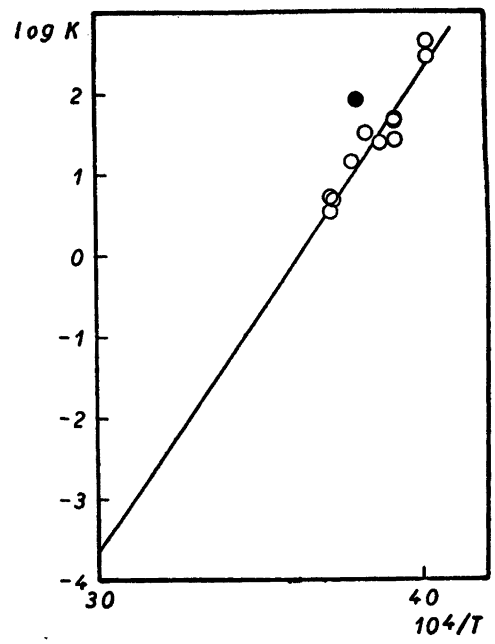

Fig. 2. Fitting a straight line to the plots of the logarithm of the equilibrium constant K as obtained from Fig. 1 and Table 1 for the computation of the enthalpy of denaturation ΔH and for reading from the line the extrapolated values of log K for computing the corrected experimental values for plotting log k(1+K)/T; see Fig. 1. The dot gives one experimental value which for statistical reasons was not regarded in fitting the straight line.

In this way the three parameters ΔH^*, ΔH and $T_{\frac{1}{2}}$ are obtained. It is shown in Fig. 3 that a line with the parameters $\Delta H^* = 4\,700$ cal/mole,

Table 2. Example of the computation of the corrections for the low temperature denaturation.

1. The abscissa values for Figs. 1 and 2. 2. The ordinata values read in Fig. 2. 3. The correction term. 4. The ordinata values for the corrected experimental plots in Fig. 1.

1	2	3	4
$10^4/T$	log K	log $(1+K)$	log $k\,(1+K)/T$
30.94	0.886 − 4	0.000	0.228 − 1
31.93	0.476 − 3	0.001	0.209 − 1
32.99	0.104 − 2	0.006	0.012 − 1
34.11	0.776 − 2	0.025	0.629 − 2
35.32	0.494 − 1	0.118	0.763 − 2
35.95	0.873 − 1	0.242	0.674 − 2
36.58	0.249	0.443	0.869 − 2
37.22	0.631	0.723	0.553 − 2
37.22	0.631	0.723	0.691 − 2
37.29	0.672	0.756	0.629 − 2

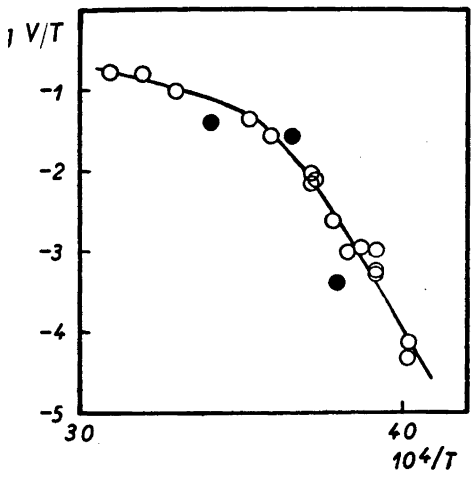

Fig. 3. The activity of lipase as measured by Sizer and Josephson [13]. The curve was calculated with the constants $\Delta H^* = 4\,700$ cal/mol., $\Delta H = -27\,000$ cal/mole, and $T_{\frac{1}{2}} = 276.4$ °K. Rings: experimental values. Dots: experimental values which due to specified reasons were not considered in calculating the constants.

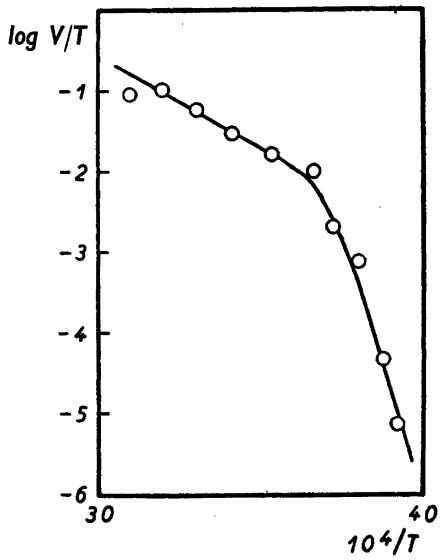

Fig. 4. The activity of saccharase as measured by Sitzer and Josephson [13]. The curve was calculated with the constants $\Delta H^* = 10\,000$ cal/mole, $\Delta H = -51\,000$ cal/mole, and $T_{\frac{1}{2}} = 269.6$ °K.

$\Delta H = -27\,000$ cal/mole, and $T_{\frac{1}{2}} = 276.4$ °K fits the experimental results closely.

Sizer and Josephson [13] also investigated the influence of the temperature on the activity of saccharase. It is shown in Fig. 4 that a line with the parameters $\Delta H^* = 10\,000$ cal/mole, $\Delta H = -51\,000$ cal/mole, $T_{\frac{1}{2}} = 269.6$ °K fits the experimental results closely.

Butler [19] measured the specific reaction velocity for the hydrolysis of bensoyl-L-arginine amide with trypsin at 6.0, 15.2, 25.5, and 37.5 °C at pH 7.8, and calculated the enthalpy of activation * on the assumption that a straight line fits the experimental results. Kavanau [17] has shown that a curve fits the experimental points much better than a straight line. Unfortunately, there are not measurements enough to enable an asymptote to be fit to the curve. ** However, we can infer that the enthalpy of activation is probably lower than $\Delta H^* = 13\,800$ cal/mole. This is in agreement with the following. Also Sizer

* Butler's value is $\Delta H^* = 14\,900$ cal/mole. However, the computation is erroneous. He seems first to have computed $\mu = 14\,305$ cal/mole, and then to have added $RT = 543$ cal/mole instead of subtracting this value. Thus, Butler's result should have been $\Delta H^* = 13\,800$ cal/mole.

** See the first foot note on p. 1708.

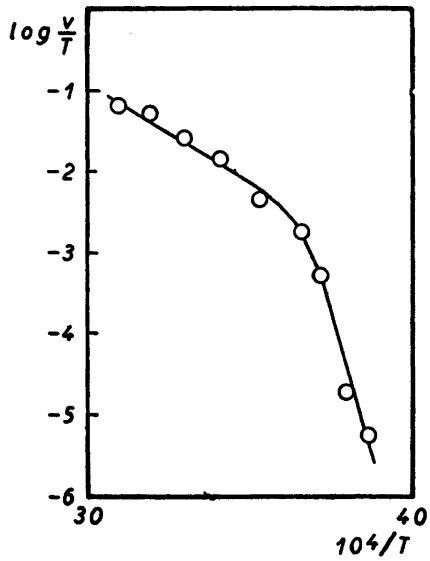

Fig. 5. The activity of trypsin as measured by Sizer and Josephson [13]. The curve was calculated with the constants $\Delta H^* = 11\,000$ *cal/mole,* $\Delta H = -53\,000$ *cal/mole, and* $T_{\frac{1}{2}} = 273.1$ *°K.*

and Josephson [13] measured the activity of trypsin at various temperatures. Their experiments were performed at pH 8.1. It is shown in Fig. 5 that a line with the parameters $\Delta H^* = 11\,000$ cal/mole *, $\Delta H = -53\,000$ cal/mole, and $T_{\frac{1}{2}} = 273.1$ °K fits the experimental results closely.

DISCUSSION

The influence of low temperatures on the activity of enzymes has been interpreted in entirely different ways by Sizer and Josephson [13] and by the present author **. In spite of this, the results of computations of characteristic constants give in both cases two values of enthalpy and one value of a characteristic temperature. The similarities and differences in the results will be discussed here.

In both treatments, one of the fitted lines concerns the same thing, the enthalpy of activation ΔH^* (or the Arrhenius activation energy μ). The line in Sizer and Josephson's treatment is directly fitted to the experimental points at high temperatures (above the break in the diagram), and in the treatment

* There may also in this case be a reversible heat denaturation for which the values at high temperatures must be corrected before the asymptote can be fitted. Anson and Mirsky [12] found that trypsin, dissolved in 0.01 N HCl is almost completely native at 40 °C but half denatured at 44 °C; however, this temperature of half denaturation was found to be very sensitive to pH and lowered by either acid or alkali.

** See foot note on p. 1702.

suggested here the line is the asymptote fitted to the experimental points. As the asymptote always has less inclination than the line fitted directly to the points, the values calculated according to Sizer and Josephson are higher than the values calculated on the basis of the theory of reversible denaturation.

The other line in the diagram of Sizer and Josephson is also assumed by them to correspond to an energy of activation μ of the enzymic process, whereas in the treatment given here the other line (Fig. 2) corresponds to the heat of the reversible denaturation of the enzyme.

Finally, the characteristic temperatures have quite different significance: in Sizer and Josephson's treatment it is a temperature at which the kinetics of the enzymic process is suddenly changed — the suggestion of such a sudden change has met several objections [15,17] — whereas in the treatment suggested here the temperature is calculated, at which half of the enzyme is reversibly denatured by the low temperature.

It is worth mentioning here that the experimental errors may by chance turn out so as to suggest the fitting of two or more straight lines in a certain way and also that it would be possible on the basis of Sizer and Josephson's hypothesis to fit straight lines in other ways in several of the published measurements (*cf.* also the corresponding problem, the influence of temperature on biological processes, reviewed by Bělehrádek [14] and discussed by Kavanau [17] and by the present author [20]).

As already mentioned, in most earlier calculations of the energy of activation μ or the enthalpy of activation ΔH^* of enzymic processes the results were founded on measurements taken in a comparatively short interval of temperature. It is therefore very likely that several of the results thus obtained are not the true values but are the sum of the true value and part of the negative value of the heat of denaturation.

It has already been mentioned that there is, at least in some cases, a reversible denaturation both at high temperatures and at low temperatures, and that the apparent values of the enzymic activity at various temperatures must first be corrected both for the low temperature and the high temperature denaturation before it is possible to draw the asymptote that corresponds to the true value of the enthalpy of activation.

In the examples of the compution given here it has been assumed that the values of enzymic activity at various temperatures published by Sizer and Josephson [13] are the limit values for high substrate concentrations. If the limit values of the enzymic activity — which are usually denoted V or k_3 — are not used, an error is introduced in the computations, due to the temperature dependence of the "Michaelis constant".

It should finally be mentioned that the denaturation both at high and at low temperatures probably depends on the pH [12], which implies that the thermodynamic values computed here are probably valid only for the particular pH at which the experiments were carried out. One other important reservation must also be forwarded: it has been assumed that the amount of native and of denatured enzyme were in equilibrium with each other; however, it is known from Kunitz [21] investigations of the soybean trypsin inhibitor that the high temperature denaturation equilibria are not reached instantly and that the reaction rate depends on the temperature and the pH.

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The Synthesis of Some Aliphatic Organosilicon Dicarboxylic Acids. II*

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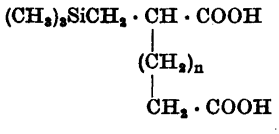
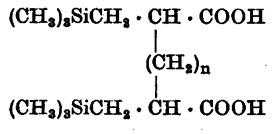
This paper deals with the preparation of some α,α'-bis-[trimethylsilylmethyl]-alkanedioic acids by way of the reaction between diethyl trimethylsilylmethylsodiomalonate and iodine and α,ω-dihalogenides. Thus, the α,α'-bis-[trimethylsilylmethyl]-glutaric, -adipic, -pimelic, and -suberic acids have been prepared, whereas the succinic acid derivative could not be synthesized by this method. The acids have all been separated into two isomers, corresponding to the *meso* and racemic forms. An anomalous cleavage of one carbethoxy group in the reaction between diethyl trimethylsilylmethylsodiomalonate and methylene bromide has been described and diethyl trimethylsilylmethyl-bromomethylmalonate has been prepared.

In a previous paper [1] was described the preparation of some α-mono-[trimethylsilylmethyl]-substituted dicarboxylic acids of the general formula I. It would be of interest from several points of view to develop a preparative route to the corresponding α,α'-bis-[trimethylsilylmethyl]-substituted dicarboxylic acids (II).



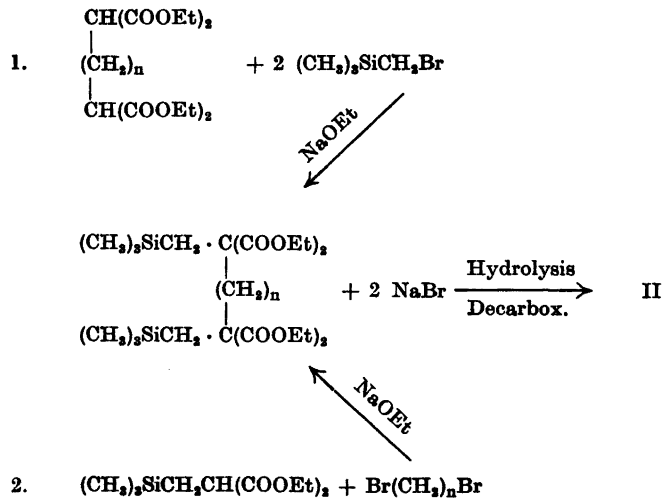
I



II

There were two possible ways of attacking the problem, which can be represented by the following formulae:

* Part I. *Acta Chem. Scand.* 8 (1954) 1183.



Of these two methods the latter seemed to be the more attractive one. Firstly, it could be expected to give better yields as there was evidence that the introduction of the trimethylsilylmethyl group into a monosubstituted malonic ester goes rather slowly. Thus, in the preparation of triethyl trimethylsilylmethylcarboxymethylmalonate (III) a much better over-all yield was obtained when the

$$(CH_3)_3SiCH_2 \cdot C(COOEt)_2CH_2 \cdot COOEt$$
III

trimethylsilylmethyl group was introduced before the carbethoxymethyl group [1]. Similarly, Sommer and Marans [2] found that it was impossible to introduce a trimethylsilylmethyl group into ethyl α-methyl- or α-ethylacetoacetate. In this case the negative result could not be explained by the nonreactivity of the halogen atom, as iodomethyltrimethylsilane was used in the experiments. A case where the nonreactivity could be explained on this basis has been described by the author [3]. Secondly, method 2 has never been studied systematically for the same alkyl-substituted malonic ester, and the synthetic possibilities of the method for the preparation of a series of α,α'-dialkyl-substituted dicarboxylic acids are therefore not satisfactorily known. Finally, this method permits a more direct study of the manner in which the trimethylsilylmethyl group differs from other groups which do not contain silicon.

Some of these aspects will be discussed more in detail under the descriptions of the reactions between the diethyl trimethylsilylmethylsodiomalonate and the individual dihalogenides.

The reaction between diethyl trimethylsilylmethylsodiomalonate and iodine

The reaction between the sodium derivatives of alkylsubstituted malonic esters and iodine has been studied in some detail by Bischoff *et al.*[4,5] They concluded that the coupling of the malonic ester residues to form the tetraester was only a side-reaction, the major part reacting according to the formula:

$$R \cdot CH(COOEt)_2 + I_2 \xrightarrow{NaOEt} RC(I) \cdot (COOEt)_2 + NaI$$

However, their results might be explained by the fact that they used equimolecular amounts of the reactants instead of the proportion 2:1 which is required for the formation of the tetraester.

When the sodium compound of diethyl trimethylsilylmethylmalonate reacted with iodine in ethanol-ether or toluene solution in the molecular proportion 2:1 a good yield of the expected tetraester was obtained. The only indication of the formation of diethyl trimethylsilylmethyliodomalonate was the appearance of minute amounts of iodine in the foreruns when the reaction product was distilled. No attempts to synthesize this iodine compound were undertaken as it could be expected to be unstable (β-silicon effect).

The best yields were obtained when the reaction was performed in toluene solution. This might be attributed to the non-solvolytic conditions, allowing only a minimum of side-reactions to occur. However, it was a very slow process to dissolve sodium cut into pieces or in the form of wire in a solution of the malonic ester in toluene or benzene. After boiling the mixture for 8 hours there still remained small fragments of sodium undissolved. This difficulty was overcome by the use of the highly reactive sodium-toluene dispersion described by Hansley[6] and Frampton and Nobis[7]. Using this dispersion the sodium dissolved in a period of 2—3 minutes and undesirable side-reactions were avoided. (All malonic ester syntheses carried out in toluene solution which are described in this paper were made according to this method).

Unfortunately, the tetraester thus prepared was very resistant to hydrolysis and, in fact, could not be hydrolysed under conditions which did not cause cleavage of methyl groups from the silicon atoms. The following conditions were tried: (1) boiling with concentrated aqueous alkali for 48 hours; (2) boiling with 10 % ethanolic alkali for 48 hours; (3) heating with 10 % ethanolic alkali at 170° in an autoclave for 24 hours. In this case probably both hydrolysis and methyl-silicon cleavage occurred, an alkali-soluble tar being obtained as the major product; (4) boiling with 64 % hydrobromic acid for 48 hours; (5) boiling with sulphuric acid of various strengths. In the last case no hydrolysis was observed at concentrations below 70 % and above this concentration methyl-silicon cleavage set in, only tar products being obtained.

The reaction between diethyl trimethylsilylmethylsodiomalonate and methylene bromide

This reaction exhibited an abnormal course as *one* carbethoxy group was eliminated when the reaction was performed in ethanolic solution, according to the formula:

$$2\ (CH_3)_3SiCH_2 \cdot CH(COOEt)_2 + CH_2Br_2 \xrightarrow{NaOEt}$$

$$(CH_3)_3SiCH_2 \cdot C(CH_2)(COOEt)_2 + (EtO)_2CO + 2\ NaBr$$

$$(CH_3)_3SiCH_2 \cdot CH(COOEt)$$

Souther [8] has described a similar reaction between diethyl phenylsodiomalonate and methylene iodide, in which case *two* carbethoxy groups were eliminated and diethyl α,α'-diphenylglutarate was formed.

In the hope of obtaining the normal product, the reaction was undertaken in toluene solution. In this case, however, no tetraester was formed but instead the diethyl trimethylsilylmethyl-bromomethylmalonate formed by the reaction

$$(CH_3)_3SiCH_2 \cdot CH(COOEt)_2 + CH_2Br_2 \xrightarrow{NaOEt}$$

$$\longrightarrow (CH_3)_3SiCH_2 \cdot C(CH_2Br)\ (COOEt)_2 + NaBr$$

This compound could also be prepared from diethyl trimethylsilylmethylsodiomalonate and methylene bromide in excess in ethanolic solution.

Finally, no tetraester was formed when reaction scheme 1 was tried. Only unreacted starting material and ethoxymethyltrimethylsilane formed according to the formula

$$(CH_3)_3SiCH_2Br + NaOEt \longrightarrow (CH_3)_3SiCH_2OEt + NaBr$$

were obtained. This was further evidence that the trimethylsilylmethyl group should be introduced first in syntheses of this type.

The triester was readily hydrolyzed by ethanolic alkali.

The reaction between diethyl trimethylsilylmethylsodiomalonate and ethylene bromide, trimethylene bromide, and tetramethylene bromide

These dihalogenides reacted with diethyl trimethylsilylmethylsodiomalonate in the proportions 1:2 in ethanolic solution to give the expected tetraesters. However, in the case of ethylene bromide the reaction had to be performed in toluene solution to obtain reasonable yields of the tetraester, as Kitzing [9] already has pointed out.

These tetraesters were readily hydrolyzed by ethanolic alkali.

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* This reaction will be more closely investigated with alkylmalonic esters in a coming paper.

The α,α'-bis-[trimethylsilylmethyl]-alkanedioic acids (II, n = 1—4)

These acids contain two identical asymmetric carbon atoms and thus must be expected to exist in *meso* and racemic forms. This was also found to be the fact and all the α,α'-bis-[trimethylsilylmethyl]-substituted dicarboxylic acids could be separated more or less readily into two forms.

The α,α'-bis-[trimethylsilylmethyl]-succinic acid could not be prepared by this method as has already been mentioned.

The crude mixture of α,α'-bis-[trimethylsilylmethyl]-glutaric acids obtained from the decarboxylation of the corresponding triacid was separated into the two forms by a simple crystallization from ethyl acetate in which solvent the high-melting isomer was much less soluble than the low-melting one. One recrystallization from ethyl acetate gave the high-melting form in the pure state, m. p. 187—188°, whereas the low-melting form required three recrystallizations from 80 % acetic acid before being obtained in a pure state, m. p. 118—120°.

Both isomers were converted into their anhydrides by boiling with acetyl chloride. The melting points of the anhydrides were 44—44.5° and 36—38°, respectively. The anhydrides were easily converted to the corresponding acids by treatment with dilute sodium hydroxide.

The two forms of the α,α'-bis-[trimethylsilylmethyl]-adipic acid were also easily separated by a simple crystallization from ethyl acetate and the final purification was achieved by recrystallizing the acids twice from 80 % acetic acid. The melting points were 122—123° and 102—104°, respectively.

However, in the case of the α,α'-bis-[trimethylsilylmethyl]-pimelic acids the difference in solubility between the isomers was rather small and a laborious and time-consuming fractionated crystallization from benzene was necessary to obtain a separation. The final purification was again performed by several recrystallizations from 80 % acetic acid. The difference in melting points was very small too, the acids melting at 91.5—93° and 87.5—89.5°, respectively. That the acids were not identical was proved by the mixed fusion method according to Kofler [10], and further by converting the acids into the dianilides, which melted at 192—193° and 162—165°, respectively.

The two α,α'-bis-[trimethylsilylmethyl]-suberic acids also presented difficulties in the separation but a four-stage fractionated crystallization from ether-petroleumether gave the isomers in an almost pure state. Final purification was performed by two recrystallizations from 80 % acetic acid. The melting points were 102—103° and 83—86°, respectively.

It would be of interest to compare the melting points of the acids prepared here with those of the corresponding α,α'-dimethyl- and α,α'-diethylsubstituted dicarboxylic acids. These data are given in Table 1.

As is seen from Table 1, the silicon-containing acids except the glutaric are in accordance with the statement made by Batzer and Wiloth [11] that α,α'-dialkylsubstituted dicarboxylic acids with an even number of carbon atoms in the chain exhibit greater differences in the melting points of the diastereoisomers than those with an odd number. In the case of the α,α'-bis-[trimethylsilylmethyl]-glutaric acid the difference in both melting points and

Table 1. Melting points of α,α'-dialkyl-substituted dicarboxylic acids.

Name of the parent dicarboxylic acid	Number of C-atoms in the chain	α,α'-substituent		
		CH_3-	C_2H_5-	$(CH_3)_3SiCH_2$-
Succinic acid	4	192° 123–124° 128°	192° 129° 119–120°	– – 187–188°
Glutaric acid	5	102–104°	93.5–94.5°	118–120°
Adipic acid	6	142° 70–72°	136° 51–53°	122–123° 102–104°
Pimelic acid	7	81–81.5° 76–76.5°	Not separated	91.5–93° 87.5–89.5°
Suberic acid	8	Not separated	Not separated	102–103° 83–86°

solubilities of the diastereoisomers was abnormally great as compared with the dimethyl- and diethyl-derivatives. Unfortunately, the succinic acid derivative was not available for comparison, and therefore conclusions regarding this anomaly could not be drawn. Other methods of preparing this acid are being investigated.

EXPERIMENTAL

All reagents used were carefully dried and fractionated in a glass-helix packed column of about ten theoretical plates. In the malonic ester syntheses precautions were taken to exclude moisture from the reaction vessels. The reactions with sodium dispersions were performed in an atmosphere of nitrogen to prevent oxidation of the sodium. Determinations of the melting points were made with a Kofler micro hot stage [10].

Preparation of sodium dispersions. A 20 % sodium dispersion in toluene was prepared by melting 50 g of sodium in 200 g of toluene and stirring the mixture for 10 minutes at 105° with a special high-speed stirrer at 4000 r.p.m.; 1 % of oleic acid was added as a stabilizer. The sodium particles of the dispersion thus prepared had an average size of about 10 μ which was sufficient for this work. The dispersion was very reactive and had to be handled with great care. It could be stored in thick-walled glass-bottles in an atmosphere of nitrogen for several months without losing its reactivity.

1,2-bis-[trimethylsilylmethyl]-1,1,2,2-tetracarbethoxyethane. A. In a 1000 ml three-necked flask equipped with a mechanical stirrer, a dropping funnel, a reflux condenser, and a thermometer reaching into the flask 4.6 g (0.2 mole) of sodium was dissolved in a mixture of 60 ml of absolute ethanol and 150 ml of absolute ether. After the addition of 49.2 g of diethyl trimethylsilylmethylmalonate the flask was cooled in an ice-bath until the temperature was below 5°. A solution of 25.4 g (0.1 mole) of iodine in 200 ml of absolute ether was added at such a rate that the temperature was kept below 10°. Each drop of iodine gave a light brown colour which disappeared almost instantaneously. The mixture was allowed to reach room temperature and finally boiled for half an hour. After cooling, 150 ml of water was added, the ethereal layer was separated and washed with water, sodium thiosulphate solution, and water again. The ether solution was dried with anhydrous magnesium sulphate, the ether distilled off and the residue distilled *in vacuo*. This method gave 15.1 g of recovered diethyl trimethylsilylmethylmalonate, slightly discoloured by iodine, and 21.5 g (0.044 mole) of the tetraester, a 63 % yield based on un-

recovered material. The tetraester was a very viscous oil which solidified completely after standing for 3 weeks at room temperature. On account of its very bad crystallizing properties it could not be made to crystallize from any solvent. B. p. 196—198°/11 mm, 171—173°/2 mm, m. p. 44—46°. (Found: C 53.7; H 8.6; Si 11.4; Calc. for $C_{22}H_{42}O_8Si_2$: C 53.8; H 8.6; Si 11.4).

B. In a 500 ml flask equipped as in the foregoing synthesis there was placed 11.5 g of a 20 % sodium-toluene dispersion (0.1 mole) together with 50 ml of sodium-dried toluene and 24.6 g (0.1 mole) of diethyl trimethylsilylmethylmalonate was added cautiously. After 5 minutes all the sodium had dissolved and a clear yellow solution resulted. The flask was cooled in an ice-bath and 12.7 g (0.05 mole) of iodine in 40 ml of toluene was added. The temperature was kept below 10°. After heating at 40° for one hour the reaction mixture was worked up as before. This procedure gave 5.6 g of the recovered malonic ester and the tetraester amounted to 14.5 g (0.030 mole), a 77 % yield, based on unrecovered malonic ester.

Attempts to hydrolyze the 1,2-bis-[trimethylsilylmethyl]-1,1,2,2-tetracarbethoxyethane. 1) 4.9 g (0.01 mole) of the tetraester was boiled for 48 hours with a solution of 6.7 g (0.12 mole) of potassium hydroxide in 20 ml of water. After cooling the mixture was extracted twice with ether and the aqueous layer was acidified with hydrochloric acid. No oil or solid could be extracted from the solution. From the ether layer 92 % of the tetraester was recovered.

2) Boiling for 48 hours with 10 % ethanolic potassium hydroxide (0.12 mole in 80 ml of ethanol) of 0.01 mole of the tetraester gave as above about 90 % of the tetraester in recovery and no alkali-soluble matter was obtained.

3) 4.9 g (0.01 mole) of the tetraester and 0.12 mole of potassium hydroxide in 80 ml of ethanol was heated in an autoclave at 150—160° for 24 hours. Working up the dark brown reaction mixture yielded 0.5 g of the tetraester and 3.4 g of a dark, alkali-soluble tar which did not solidify even upon prolonged standing in a vacuum desiccator over concentrated sulphuric acid.

4) 4.9 g of the tetraester + 35 ml of 66 % hydrobromic acid was boiled in a flask equipped with a 60 cm Vigreux column for 48 hours. No formation of ethyl alcohol or ethyl bromide could be detected, and almost all the tetraester was recovered.

5) When boiling the tetraester with sulphuric acid of various concentrations no effect was observed at concentrations below 70 % even after several days' of boiling. At 70 %, however, small amounts of methane were formed but no hydrolysis was observed.

4.9 g of the tetraester was boiled with 20 ml of 75 % sulphuric acid for 15 minutes. Methane was evolved. The reaction mixture was diluted with water and a sticky solid was formed which was filtered and dried. On titration with standard alkali solution it showed an equivalent weight of 155 and it had no definite melting point.

1,3-bis-[trimethylsilylmethyl]-1,1,3-tricarbethoxypropane. A. In the apparatus mentioned previously 11.5 g (0.5 mole) of sodium was dissolved in 150 ml of absolute ethanol. 123 g (0.5 mole) of diethyl trimethylsilylmethylmalonate was added, followed by the dropwise addition of 43.5 g (0.25 mole) of methylene bromide at a temperature of about 80°. After refluxing for twenty hours the reaction mixture was neutralized with some glacial acetic acid and the alcohol distilled off on a water-bath through a short column. The residue was washed with water and dried with anhydrous magnesium sulphate. The following fractions were obtained after distillation *in vacuo:* I, b. p. 65—68°/100 mm, 14.5 g; II, b. p. 88—90°/2 mm, 35.0 g; III, b. p. 158—161°/2 mm, 67.3 g. Fraction I was redistilled, b. p. 124—125°/760 mm, and identified as diethyl carbonate by means of refractive index and density, n_D^{20} 1.3841, and d_3^{20} 0.9753, (data from handbook: n_D^{20} 1.3845; d_4^{20} 0.9751). Fraction II consisted of diethyl trimethylsilylmethylmalonate while III was the triester, n_D^{20} 1.4527, d_4^{20} 0.9874. The yield based on unrecovered material was 85 %. (Found: C 55.7; H 9.4; Si 12.9; r_D 0.2736. Calc. for $C_{20}H_{40}O_6Si_2$: C 55.5; H 9.3; Si 13.0; r_D 0.2742).

B. In the same apparatus 0.2 mole of sodium was dissolved in 70 ml of absolute ethanol. 0.1 mole of 1,1,3,3-tetracarbethoxypropane was added followed by 0.2 mole of bromomethyltrimethylsilane. After refluxing for 24 hours 150 ml of water was added, the organic layer was washed with water 3 times and finally dried with anhydrous magnesium sulphate. Distillation yielded 10.5 g (0.080 mole) of ethoxymethyltrimethylsilane, b. p. 101—103°/760 mm, n_D^{20} 1.3935, and d_4^{20} 0.7538 (handbook: n_D^{20} 1.3940, d_4^{25} 0.755), a

40 % yield, and most of the 1,1,3,3-tetracarbethoxypropane in recovery. No higher-boiling fractions were obtained.

Diethyl trimethylsilylmethyl-bromomethylmalonate. A. In ethanolic solution. In the usual apparatus 4.6 g (0.2 mole) of sodium was dissolved in 70 ml of absolute ethanol and a mixture of 49.2 g (0.2 mole) of diethyl trimethylsilylmethylmalonate and 69.6 (0.4 mole) of methylene bromide was added slowly. The mixture was then boiled under reflux for 2 hours and worked up as before, giving 44.4 g of diethyl trimethylsilylmethylbromomethylmalonate, b. p. 153—156°/15 mm, n_D^{20} 1.4603, d_4^{20} 1.1884, a 65 % yield. (Found: Br 23.3; Si 8.3; r_D 0.2306. Calc. for $C_{12}H_{23}O_4BrSi$: Br 23.6; Si 8.3; r_D 0.2305).

B. In toluene solution. In the usual apparatus 11.5 g of a 20 % sodium-toluene dispersion (0.1 mole) was placed together with 50 ml of toluene. 24.6 g (0.1 mole) of diethyl trimethylsilylmethylmalonate was added carefully. When the sodium had dissolved 0.05 mole of methylene bromide was added dropwise and the reaction mixture was refluxed for 8 hours. After the usual treatment 6.0 g of diethyl trimethylsilylmethylbromomethylmalonate was obtained, b. p. 116—117°/2 mm, n_D^{20} 1.4597, and d_4^{20} 1.1883, a 35 % yield (based on methylene bromide). No higher-boiling fractions were obtained.

α,α'-bis-[trimethylsilylmethyl]-glutaric acid. 61.5 g (0.142 mole) of the 1,3-bis-[trimethylsilylmethyl]-1,1,3-tricarbethoxypropane was hydrolyzed by boiling with excess ethanolic potassium hydroxide for 20 hours. To the solution was added 200 ml of water and the ethanol was distilled off. Upon acidification with hydrochloric acid the triacid precipitated. It was filtered, washed thoroughly with water and finally air-dried. A small quantity was recrystallized from acetone, m. p. 163—169° (decomp.). (Found: C 47.9; H 8.2; Si 16.2. Calc. for $C_{14}H_{28}O_6Si_2$: C 48.2; H 8.1; Si 16.1).

The main bulk of the crude triacid, 48.0 g (0.138 mole), was decarboxylated by heating to 180—200° for 15 minutes. After cooling the acid mixture was dissolved in 350 ml of boiling ethyl acetate, filtered and allowed to stand for 6 hours. The crystals formed (19.5 g) were filtered and washed with ethyl acetate, m. p. 185—187°. The filtrate was evaporated to a volume of 40 ml, whereafter a new portion of crystals (4.0 g) was obtained, m. p. 183—187°. These two fractions were combined and recrystallized once from ethyl acetate, m. p. 187—188°. The mother liquor was evaporated to dryness and the residue was recrystallized 3 times from 80 % acetic acid, yielding 9.5 g of the low-melting form, m. p. 118—120°. The yields were 56 and 23 %, respectively. (Found for the high-melting form: C 51.2; H 9.3; Si 18.5; equiv. wt. 152.7. Found for the low-melting form: C 51.1; H 9.1; Si 18.5; equiv. wt. 152.1. Calc. for $C_{13}H_{28}O_4Si_2$: C 51.3; H 9.3; Si 18.5; equiv. wt. 152.3).

Anhydrides of the glutaric acids. 0.01 mole of each acid was refluxed with 5 ml of acetyl chloride for 3 hours. The excess acetyl chloride was distilled off and the residue distilled *in vacuo*. Both acid anhydrides boiled at 155—156°/2.5 mm and were formed in about 85 % yields. They were viscous oils which soon solidified. The anhydride of the high-melting acid melted at 44.5—45° and that of the low-melting one at 36—38°. No attempts to recrystallize the anhydrides were made. (Found for the high-melting form: C 54.4; H 9.1; Si 19.6; equiv. wt. 143.6. Found for the low-melting form: C 54.4; H 9.1; Si 19.5; equiv. wt. 143.2. Calc. for $C_{13}H_{26}O_3Si_2$: C 54.5; H 9.1; Si 19.6; equiv. wt. 143.3).

1,4-bis-[trimethylsilylmethyl]-1,1,4,4-tetracarbethoxybutane. This compound was prepared in the usual manner from 23.0 g of 20 % sodium dispersion (0.2 mole) in 100 ml of toluene, 49.2 g (0.2 mole) of diethyl trimethylsilylmethylmalonate, and 18.8 g (0.1 mole) of ethylene bromide. The reaction mixture was refluxed for 20 hours before working up. 8.5 g of the malonic ester was recovered and the tetraester amounted to 21.2 g, b. p. 188—191°/2.5 mm, a 50 % yield (based on unrecovered material). The distillate soon solidified and had the melting point 53—54°. (Found: C 55.6; H 9.0; Si 10.8. Calc. for $C_{34}H_{66}O_8Si_2$: C 55.6; H 8.9; Si 10.8).

α,α'-bis-[trimethylsilylmethyl]-adipic acid. 31.2 g (0.060 mole) of the tetraester was hydrolyzed with ethanolic potassium hydroxide as before. A small quantity of the tetraacid was recrystallized from acetone-petroleumether, m. p. 202° (decomp.). (Found: C 47.4; H 7.4; Si 13.8. Calc. for $C_{16}H_{30}O_8Si_2$: C 47.3; H 7.4; Si 13.8).

24.0 g (0.059 mole) of the tetraacid was decarboxylated at 200—210° for 20 minutes. The crude acid mixture was dissolved in 60 ml of boiling ethyl acetate, filtered, and allowed to cool. After 3 hours the crystals (5.9 g) were filtered and washed with ethyl acetate, m. p. 115—120°. After 2 recrystallizations from 80 % acetic acid the acid was

obtained in a pure state, 4.5 g, m. p. 122—123°. The mother liquor was evaporated to dryness and the residue recrystallized twice from 80 % acetic acid. The low-melting form was thus obtained pure, 9.5 g, m. p. 102—104°. The yields were 24 and 51 %, respectively. (Found for the high-melting form: C 52.8; H 9.6; Si 17.6; equiv. wt. 159.3. Found for the low-melting form: C 52.8; H 9.5; Si 17.6; equiv. wt. 159.4. Calc. for $C_{14}H_{30}O_4Si_2$: C 52.8; H 9.5; Si 17.6; equiv. wt. 159.3).

1,5-bis-[trimethylsilylmethyl]-1,1,5,5-tetracarbethoxypentane. This synthesis was performed as a conventional malonic ester synthesis from 4.6 g (0.2 mole) of sodium dissolved in 70 ml of absolute ethanol, 49.2 g (0.2 mole) of diethyl trimethylsilylmethylmalonate, and 20.2 g (0.1 mole) of trimethylene bromide. The mixture was boiled for 2 hours. 39.0 g (0.073 mole) of the expected tetraester was obtained, b. p. 202—203°/2 mm, m. p. 67—68°. 10.0 g of the malonic ester was recovered. The yield based on unrecovered material was 91 %. (Found: C 56.5; H 9.2; Si 10.5. Calc. for $C_{25}H_{48}O_8Si_2$: C 56.4; H 9.1; Si 10.5).

α,α'-bis-[trimethylsilylmethyl]-pimelic acid. The tetraester was hydrolyzed as before giving the corresponding tetraacid in 97 % yield. A small amount was recrystallized from acetone-petroleumether, m. p. 180—183° (decomp.). (Found: C 48.7; H 7.8; Si 13.5. Calc. for $C_{17}H_{32}O_8Si_2$: C 48.5; H 7.7; Si 13.5).

46.0 g (0.110 mole) of the tetraacid was decarboxylated at 180—200° for 20 minutes yielding 36 g (0.108 mole) of the crude pimelic acids. This mixture was submitted to a four-stage fractionated crystallization from benzene. The fractions could then be combined to give 2 main bulks of the melting points 82—89° (13.5 g) and 87—93° (16.2 g). Each fraction was recrystallized 4 times from 80 % acetic acid to give the pure acids, m. p. 87.5—89.5° (8.0 g) and 91.5—93° (13.1 g). The eutectic melting point was determined by the mixed fusion method and was 65°. The yields of the pure acids were 22 and 36 %, respectively. (Found for the acid with m. p. 91.5—93°: C 54.3; H 9.7; Si 16.9; equiv. wt. 166.4. Found for the acid with m. p. 87.5—89.5°: C 54.2; H 9.7; Si 16.9; equiv. wt. 166.6. Calc. for $C_{15}H_{32}O_4Si_2$: C 54.2; H 9.7; Si 16.9; equiv. wt. 166.3).

Preparation of the anilides. The anilides were prepared by refluxing 0.6 g of each of the acids with 1 ml of thionyl chloride for 15 minutes, evaporation of the excess thionyl chloride, and addition of 0.8 g of aniline in 10 ml of benzene. The mixture was boiled for 10 minutes, water added, the benzene layer separated, and washed twice with dilute hydrochloric acid and finally with water. The benzene was distilled off and the residue recrystallized 4 times from 70 % ethanol. The high-melting acid gave an anilide melting at 192—193°, while the low-melting one gave an anilide melting at 162—165°. (Found for the high-melting isomer: C 67.2; H 8.7; Si 11.5. Found for the low-melting isomer: C 67.0; H 8.7; Si 11.5. Calc. for $C_{27}H_{42}O_2N_2Si_2$: C 67.2; H 8.8; Si 11.6).

1,6-bis-[trimethylsilylmethyl]-1,1,6,6-tetracarbethoxyhexane. In the usual apparatus 4.6 g (0.2 mole) of sodium was dissolved in 70 ml of absolute ethanol, and 49.2 g (0.2 mole) of diethyl trimethylsilylmethylmalonate was added. 21.6 g (0.1 mole) of tetramethylene bromide was added dropwise at such a rate that the mixture refluxed gently. After the addition the reaction mixture was boiled under reflux for 2 hours. Then it was neutralized with a few drops of glacial acetic acid, the ethanol distilled off and 150 ml of water added. The oil solidified immediately and was filtered, washed with water and air-dried. It was sufficiently pure for direct use in the preparation of the suberic acids. The yield was 45.4 g (0.083 mole), 83 %. A small quantity was recrystallized from petroleum-ether, m. p. 93—94°. (Found: C 57.1; H 9.2; Si 10.3. Calc. for $C_{26}H_{50}O_8Si_2$: C 57.1; H 9.2; Si 10.3).

α,α'-bis-[trimethylsilylmethyl]-suberic acid. The tetraester was hydrolyzed as before yielding 98 % of the corresponding tetraacid. A small quantity was recrystallized from acetone-petroleumether, m. p. 205—210° (decomp.). (Found: C 50.0; H 7.9; Si 13.0. Calc. for $C_{18}H_{34}O_8Si_2$: C 49.8; H 7.9; Si 12.9).

33.0 g of the crude acid mixture obtained after decarboxylation at 210—220° for 10 minutes was submitted to a four-stage fractionated crystallization from ether-petroleum-ether. The fractions were combined into 2 main bulks of the melting points 93—100° (8.0 g) and 81—87° (16.3 g). The high-melting portion required 2 recrystallizations from 80 % acetic acid before being obtained pure, m. p. 102—103° (5.5 g) whereas the low-melting one required 4 recrystallizations from the same solvent, m. p. 83—86° (11.2 g). The yields of the pure acids were 17 and 33 %, respectively. (Found for the high-melting form: C 55.3; H 9.8; Si 16.3; equiv. wt. 173.1. Found for the low-melting form: C 55.5;

H 9.9; Si 16.2; equiv. wt. 172.9. Calc. for $C_{16}H_{34}O_8Si_2$: C 55.4; H 9.9; Si 16.2; equiv. wt. 173.3).

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Short Communications

Infrared Absorption Spectra of Aluminium Soaps of Rosin Acids

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Harple, Wiberley and Bauer [1] have studied the infrared spectra of aluminium soaps of fatty acids synthesized by an aqueous metathesis method. They found that both aluminium soaps with one mole of aluminium to two moles of fatty acid (disoap) and those with one mole of aluminium to one mole of fatty acid (monosoap) exist as distinct chemical compounds. They were unable, however, to establish the existence of a trisoap. The spectra of the disoaps contained a free hydroxyl band (2.7 μ), those of the monosoaps a band of bonded hydroxyl (3.0 μ).

The present paper gives the results of an infrared spectroscopic study of the structure of aluminium soaps of rosin acids. The substances investigated were the aluminium monosoaps of tetrahydroabietic and *iso*dextropimaric acids and five samples of the former acid containing different amounts of aluminium from the nearly pure acid to the monosoap. For comparison, aluminium monomyristate was also investigated.

Experimental. Monolayer studies of various rosin acids [2], purified tall oil rosin acid (a mixture of rosin acids) [3-5] and myristic acid [6-9] have shown that these acids react with aluminium ions under suitable conditions when they are spread in a monomolecular layer on the surface of an aluminium salt solution. At certain pH values and aluminium concentrations aluminium soaps are produced in which the molar ratio of aluminium to acid is unity or less than unity. (The compositions of the monolayers were determined on the dried monolayer substances after they had been skimmed from the substrate surface.) The samples studied in this investigation were prepared on the basis of this experience. The substrates from which the monolayer substances were collected by skimming were 0.001 M in potassium aluminium sulphate; in the preparation of the monosoaps the pH was 4.30 and in the preparation of the five samples of tetrahydroabietic acid containing different amounts of aluminium the pH's were 3.70, 3.84, 4.00, 4.12 and 4.24. The compositions of the samples were checked by micro combustion analyses *, which gave a carbon content of 67.2 wt % for the monosoap of the tetrahydroabietic acid (calculated carbon content of dibasic aluminium monosoap, 66.3 %, of monobasic aluminium disoap, 74.3 %, and of aluminium trisoap, 77.3 %) and carbon contents 77.8, 76.2, 73.9, 71.6 and 69.1 % for the five samples (calculated carbon content of pure tetrahydroabietic acid, 78.3 %). The samples on which the analyses and spectroscopic studies were made were previously dried at 80—90° C in a vacuum over phosphorus pentoxide.

The infrared absorption spectra were recorded with a double beam spectrophotometer ** of the type described by Hornig, Hyde and Adcock [10]. The samples were prepared for the measurements by the potassium bromide technique.

Results and conclusions. The infrared absorption spectra of the aluminium monosoaps of the tetrahydroabietic and *iso*dextropimaric acids showed the following strong (s) and medium (m) absorption bands (in μ): 3.0 (m), 3.4—3.5 (s), 5.9 (m), 6.3 (s), 6.8—6.9 (s), 6.9—7 (s), 7.2 (m), 7.3 (m), 10.0—10.2 (s), 11.0 (s) (only for

* These analyses were performed in the Microanalytical Laboratory of the Institute of Medical Chemistry of Uppsala University.

** Built by Dr. M. Skog.

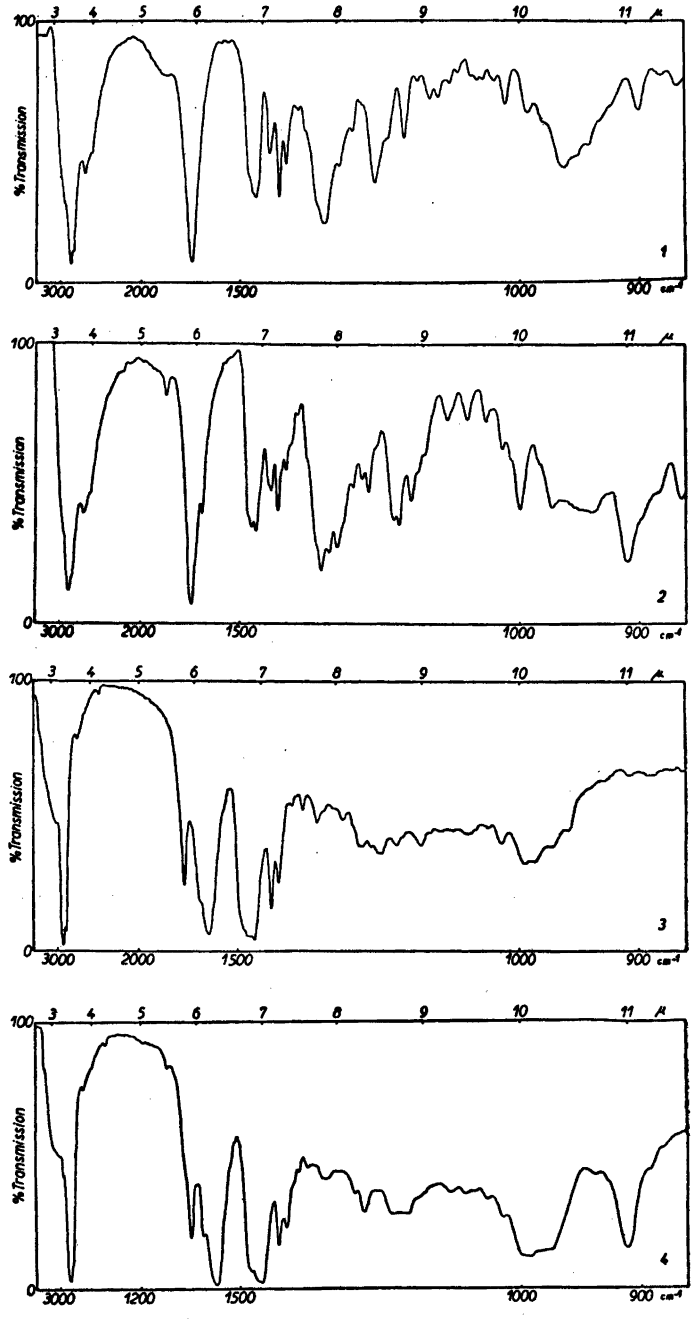

Fig. 1. Infrared absorbtion spectra of 1) tetrahydroabietic acid, 2) isodextropimaric acid, 3) aluminium monosoap of tetrahydroabietic acid, 4) aluminium monosoap of isodextropimaric acid.

the monosoap of *iso*dextropimaric acid), 12.9—13.0 (m). The bands at 3.4—3.5, 6.8—6.9, 7.2 and 7.3 μ are due to the hydrocarbon part of the rosin acid molecule (these bands appear also in the spectra of the pure rosin acids), that at 3.0 μ to associated hydroxyl groups, the bands at 6.3, 6.9—7 and 10.0—10.2 μ to the carboxylate group of the aluminium soap. With the exception of the band 5.9 μ, which is due to the presence of small amount of unreacted acid in the sample (the unreacted acid gives weak absorption bands also at other wave lengths) and the band at 12.9—13.0 μ, the spectra are in good agreement with that for aluminium monomyristate and those of the aluminium monosoaps of α-ethylcaproic and lauric acids recorded by Harple, Wiberley and Bauer [1]. From these results it may be concluded that the aluminium monosoaps of the rosin acids exist as definite chemical compounds. Other evidence favouring this conclusion has already been presented earlier [3, 9, 5].

The spectra of the samples of tetrahydroabietic acid containing different amounts of aluminium exhibited the following changes with increasing aluminium content. The band at 3.0 μ appears while at the same time the band for dimeric carbocylic acid (3.8—4 μ) becomes weaker. The very strong band of the C = O vibration absorption (5.9 μ) is suppressed to a small peak. Bonds at 6.3 and 6.9—7 μ due to carboxylate groups appear already at a very low aluminium content (in the sample collected at pH 3.84). The bands at 7.1, 7.8, and 10.5 μ typical of carboxylic acids are greatly reduced in intensity. None of the recorded spectra revealed an absorption band at 2.7 μ characteristic of an aluminium disoap. It thus appears that no aluminium disoap of the type found with fatty acids is formed. The samples of tetrahydroabietic acid containing less aluminium than the amount corresponding to a monosoap involves a system composed of only two components, the aluminium monosoap and rosin acid. This conclusion is confirmed by the observed absorption due to the free acid in the spectra of the samples of high aluminium content *.

The band at 10.0—10.2 μ is assumed to be due to the Al-O linkage, the band is found also in the spectra of the different aluminium soaps of the fatty acids investigated by Harple, Wiberley and Bauer [1].

A detailed report of this investigation will be published later.

This study has been conducted at the Institute of Medical Chemistry of the University of Uppsala. I am indebted to Dr. Ingrid Fischmeister for assistance with the spectrophotometric measurements and for discussions.

I am grateful to Dr. C. G. Harris of Hercules Powder Company, Wilmington, USA, for samples of tetrahydroabietic and *iso*dextropimaric acids.

My visit to Sweden has been made possible by a grant from the *Committee for the Advancement of Natural Sciences* appointed by the Finnish Government.

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* Back and Steenberg [9] have isolated a compound corresponding to aluminium disoap from dried aluminium soap precipitates of abietic acid by extraction with anhydrous acetone.

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A New Type of Spectrometer for Determination of the Refractivity of Liquids and Gases

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Fig. 1 shows the basic design of a spectrometer (horizontal plane). Light from the source *A* passes the vertical slit *B* and the lens *C* and passes the vertical prism *D* as a beam which is parallel in the horizontal plane. After passing the prism *D* the light is focussed by the lens *E* at *F* and finally refocussed by the lenses *G* and *H* on the photographic plate *I*.

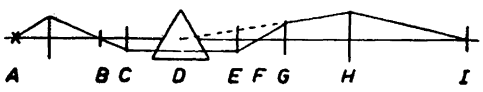

Fig. 1. Diagram of spectrometer in the horizontal plane. The lenses E *and* G *are cylindrical with vertical axis. In the vertical plane the image of the prism* D *through the lense* H *falls on* I *as indicated by the dotted line.*

The horizontal position of the vertical image of the slit on the photographic plate will depend on the refractivity of the prism *D*. The vertical prism *D* may be hollow and filled with the sample to be examined.

The lenses *E* and *G* may be cylindrical with vertical axis in which case the system will be strongly astigmatic. If the image of the prism *D* by the lens *H* in the vertical plane then falls on the photographic plate every level of the vertical prism will correspond to a definite level on the photographic plate.

If the prism contains an heterogeneous liquid — for example pure water floating on a denser sugar-water solution — the place in which the light reaches the photographic plate at any horizontal level will depend on the refractivity of the liquid at the corresponding level inside the prism, and the picture obtained on the plate will be of the kind shown in Fig. 2.

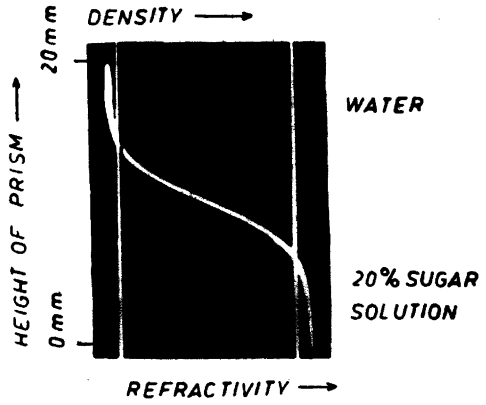

Fig. 2. Photograph taken at I *(Fig. 1) with layers of water and sugar solution inside the hollow prism* D *(Fig. 1). The effect of the diffusion of sugar into water is made visible by the sigmoid curve. The vertical lines are for orientation of the curve.*

In the case corresponding to Fig. 2 a 60° prism was used, having sides of 5 mm and a height of 20 mm. Fig. 2 is the very first picture obtained with the arrangement just described. There is little doubt that better results will be obtained later.

We wish to stress that the optical system on the light source side of the slit also has to be astigmatic. In the horizontal plane the light from the lamp *A* should be collected at the slit *B*, in the vertical plane at the prism *D*.

Cand. mag. Bjørn B. Hansen has been most active by assembling the existing apparatus and has also done the pre-calculation of the important lenses. Together we will publish more detailed descriptions of the apparatus.

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Ageratochromene, a Heterocyclic Compound from the Essential Oils of some *Ageratum* Species

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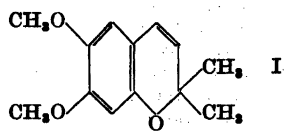
During researches carried out in this laboratory on acetylenic compounds from *Compositae*, some aromatic substances have been isolated. From the essential oils of *Ageratum mexicanum* Sims. and *A. conyzoides* L. a crystalline compound (I) $C_{13}H_{16}O_3$, for which we propose the name ageratochromene, was obtained. It had m.p. 47.5° (corr.) and was optically inactive (Found: C 70.8; H 7.35. Calc. for $C_{13}H_{16}O_3$: C 70.9; H 7.35), λ_{max} 2 800, 3 230 Å (ε_{max} 5 500 and 9 300, respectively). The infra-red spectrum revealed the aromatic character, the presence of methyl and ether groups and the absence of any carbonyl or hydroxyl functions. Catalytic hydrogenation furnished a dihydrocompound (II), m.p. 60° (corr.), λ_{max} 2 930 Å (ε_{max} 6 400). The U. V.-spectra of (I) and (II) indicated the presence of a benzene nucleus conjugated with one double bond; it then follows that (I) must be bicyclic. In contrast to (I) the dihydrocompound (II) gave a satisfactory result in the methoxyl determination. (Found: MeO 28.5. Calc. for $C_{13}H_{18}O_3$ (2 MeO): MeO 27.9). Oxidation of (I) with OsO_4 gave a glycol (III), m. p. 129.5° (corr.) (Found: C 61.35; H 7.1. Calc. for $C_{13}H_{18}O_5$: C 61.4; H 7.15). λ_{max} 2 925 (ε_{max} 4 300). Chromium trioxide oxidation of (III) gave a compound (IV) $C_{13}H_{16}O_5$, m. p. 116–118.5° (corr.). (Found: C 61.2; H 6.7 $C_{13}H_{16}O_5$ requires C 61.9; H 6.4). According to a strong I.R.-peak at 1 682 cm^{-1} (IV) contains a carbonyl group conjugated with an aromatic ring. The U.V.-spectrum, λ_{max} 2 420, 2 745, 3 375, 3 483 (ε_{max} 55 200, 13 500, 7 600 and 6 500, respectively) confirms this. Oxidation of (III) with lead tetra-acetate afforded another substance (V) $C_{13}H_{16}O_5$, m. p. 101.5–102° (corr.) (Found: 61.5; H 6.5; MeO 25.1. $C_{13}H_{16}O_5$ requires C 61.9; H 6.4; MeO 24.6), λ_{max} 2 730, 3 160 Å (ε_{max} 15 200 and 7 700, respectively). According to the U.V.-spectrum (V) should have one carbonyl group conjugated with the benzene ring, and this was confirmed by an infra-red maximum at 1 676 cm^{-1}. The infra-red spectrum also showed a strong peak at 1 740 cm^{-1} indicating another carbonyl function coinciding with the standard value for an isolated ester group. From this indication (I) was supposed to be a 1 *H*-2-benzopyran derivative and should give a dimethoxyphthalic acid when submitted to a further oxidative degradation. All experiments in this direction were unsuccessful.

Control measurements of phenoxyacetone gave an infra-red maximum at 1 736 cm^{-1}, $\Delta\nu$ = 16 cm^{-1}. For phenoxyacetaldehyde this maximum was shifted to 1 745 cm^{-1}, $\Delta\nu$ = 13 cm^{-1}. This indicated that the 1 740 cm^{-1} maximum of (V) might belong to an isolated carbonyl group and that it was displaced from its ordinary position by the presence of an α-phenoxy-residue, compare [1].

(V) could be oxidized further with potassium permanganate to a monocarboxylic acid (VI) $C_{13}H_{16}O_6$, m. p. 118–119° (corr.) (Found: C 58.0; H 6.0. Calc. for $C_{13}H_{16}O_6$: C 58.2; H 6.0), λ_{max} (in ethanol) 2 745, 3 320 (ε_{max} 8 500 and 5 400, respectively) and a dicarboxylic acid (VII) $C_{13}H_{16}O_7$, m. p. 157–159.5° (corr.) (Found: C 54.6; H 5.45. Calc. for $C_{13}H_{16}O_7$: C 54.9; H 5.65), λ_{max} (in ethanol) 2 570, 3 000 (ε_{max} 12 900 and 7 100 respectively). The double bond conjugated with the benzene ring therefore would be of the type $-CH=CH-$. As the glycol (III) gives a monoacetate (VIII), a monoketone (IV) and a monocarboxylic acid (VI), the carbon atom next to the double bond might be substituted in a manner as to give an appreciable steric hindrance. The infra-red spectra of (I) and all its derivatives showed a splitting of the methyl band into two well resolved maxima at 1 383 and 1 363 cm^{-1}. A *gem.*dimethyl group was probable, and (I) might be supposed to be a dimethoxy-2:2-dimethyl-chromene. The infra-red spectra of (I) and its derivatives further indicated a 1,2,3,5- or a 1,2,4,5-substituted benzene compound. The 5:7-dimethoxy-2:2-dimethyl-chroman (IX) had been synthesized by Alexander Robertson *et al.*[2] and (IX) was stated to be a liquid. A resynthesis confirmed this. The infra-red spectrum of (IX) showed many similarities with that of (II), but also the non-identity of the two substances. 6:7-Dimethoxy-2:2-dimethyl-chroman (X) was then synthesized following an analogous procedure, and (X) turned out to be iden-

tical with (II) according to m. p., mixed m.p. and I.R.-spectrum. Ageratochromene is therefore 6:7-dimethoxy-2:2-dimethyl-chromene.



I

The 2:2-dimethyl-chromene skeleton has been found in other natural compounds *e. g.* deguelin, toxicarol, xanthoxyletin, xanthyletin [3] and evodionol [4]. So far ageratochromene is the first compound of this class isolated from a plant belonging to the *Compositae*. Ageratochromene has the same type of benzene substitution as ayapin [5] (6:7-methylenedioxycoumarin) isolated from the closely related plant *Eupatorium ayapana* Vent. (*Ageratum* and *Eupatorium* both belong to subtribus Ageratinae, tribus Eupatorieae), scoparone [6] (6:7-dimethoxy-coumarin) isolated, *e. g.*, from the composite plants *Artemisia scoparia* and *A. capillaris*, and cichoriin [7] (6-hydroxy-7-glucosido-coumarin) isolated from the flowers of chicory.

Grants from *Norges Almenvitenskapelige Forskningsråd* are gratefully acknowledged.

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Effect of Cobalt and Iron on Riboflavin Production by *Candida guilliermondia*

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The yeast *Candida guilliermondia*, which is capable of outstanding synthesis of riboflavin, was first investigated by Burkholder [1]. Tanner *et al.*[2] found that iron, if present in the medium at a concentration of 100 μg/l, sharply reduced the riboflavin production of *C. guilliermondia*. The optimum iron concentration, according to these authors, is 5—10 μg/l. Earlier Arzberger [3] had shown that iron and cobalt, at a concentration of 3.2 mg/l, reduced the riboflavin production of *Clostridium acetobutylicum*, while zinc, copper, and lead had no influence.

In the present work it is shown that cobalt in a concentration of 10^{-4} M (5.9 mg/l) considerably enhances the riboflavin production of *C. guilliermondia* and shifts the optimum iron concentration to about 10^{-5} M (560 μg/l).

Methods. The *C. guilliermondia* strain used in this work was originally sent to the Centraalbureau voor Schimmelcultures, Baarn, Holland, by P. Burkholder, as synthesizing riboflavin. For the present work it was obtained from Mrs. June Robson, Isotope Division, A.E.R.E., Harwell, England, to whom I wish to express my thanks. The yeast was cultivated in the following medium: glucose 30 g, $(NH_4)_2HPO_4$ 3 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, biotin 5 μg, and water 1 000 ml. The pH was adjusted to 4.5. All chemicals used were of analytical grade and the water was distilled twice in a quartz glass apparatus. Iron was not removed from the medium nor was the iron content of it determined. The yeast was cultivated at 30° C with powerful aeration. The riboflavin produced was determined by direct spectrophotometry of the centrifuged medium.

Results. *C. guilliermondia* was grown in the nutrient medium with different amounts of cobalt sulphate added. Samples were taken after 1, 5, 24, and 30 hours. After 1 and 5 hours no riboflavin could be detected in the medium. The amounts of

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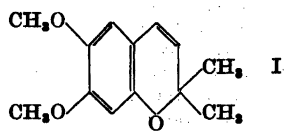

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riboflavin produced in 24 and 30 hours can be seen from Fig. 1. The curves show a very distinct maximum for riboflavin production at a cobalt concentration of 10^{-4} M. Even at a concentration of 10^{-2} M cobalt inhibits the growth of the yeast and at 10^{-3} M does so to some extent, but at 10^{-4} M does not affect growth.

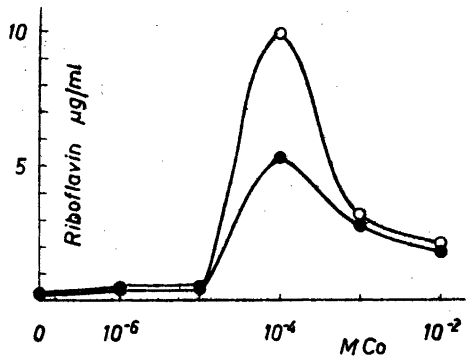

Fig. 1. Effect of cobalt on riboflavin production by C. guilliermondia. *Riboflavin estimated after 24 hours' (●) and 30 hours' (○) growth.*

Some other divalent cations and molybdate were tested at concentrations of 10^{-4} M. The only one of these, apart from cobalt, causing a sligth increase in the riboflavin production was zinc (Table 1).

Table 1. Effect of some ions on riboflavin production at concentrations of 10^{-4} M. Riboflavin estimated after 20 hours' growth.

Ion added	Riboflavin in the medium µg/ml
None	2.6
Co++	13.6
Mn++	0.3
Zn++	4.7
Cu++	0.0
MoO_4^{--}	0.0

In another experiment the yeast was grown in a medium containing 2×10^{-4} M cobalt and with different amounts of iron added (Table 2). Controls with no cobalt showed that these additions of iron completely inhibited the production of riboflavin. In the cobalt-containing medium the highest riboflavin production was obtained when 10^{-5} M of iron was added. The addition of 2×10^{-4} M of cobalt thus shifts the optimum iron concentration from about 10^{-7} M to 10^{-5} M. It seems likely that there is competition between cobalt and iron and that the action of cobalt on riboflavin production is due, at least in part, to this competition.

Table 2. Effect of cobalt and iron on riboflavin production. Riboflavin estimated after 30 hours' growth.

Cobalt added M	Iron added M	Riboflavin in the medium mg/g yeast (dry matter)
0	0	1.5
0	10^{-5}	0.0
0	10^{-3}	0.0
2×10^{-4}	0	2.7
2×10^{-4}	10^{-5}	3.9
2×10^{-4}	10^{-3}	1.0

Note. Hickey (*U. S. Patent* 2 667 445 (1954); Ref. in *Chem. Abstracts* 48 (1954) 6 084) has found that 10 mg/l of cobalt increases the riboflavin production of *Ashbya gossypii* by about 60 %, while 50 mg/l of cobalt reduces it by about 70 %.

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The Chemistry of the Natural Order Cupressales. XV *. Heartwood Constituents of *Austrocedrus chilensis* (D.Don), Florin et Boutelje (= *Libocedrus chilensis* (D.Don) Endl.)

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Most of the genera of the family Cupressaceae belong either to the northern or to the southern hemisphere but one; *Libocedrus sensu lato* has a strange distribution, occurring along the coasts of the whole Pacific.

According to modern botanical views this genus, however, does not constitute a natural group and at present the following genera are recognised: *Heyderia* (northern hemisphere), *Pilgerodendron*, *Austrocedrus*, *Papuacedrus* and *Libocedrus sensu stricto* (all southern hemisphere).

Recently Corbett and Wright [1] reported that they had been unable to isolate any characteristic heartwood constituents from *Libocedrus Bidwillii* Hook. *(L. s. str.)*, New Zealand, which is in agreement with unpublished results from this laboratory. (Similar results were also obtained with the Tasmanian *Diselma Archeri* Hook.fil. also of the Cupressaceae — B. Thomas, unpublished).

Zavarin and Anderson [2] found in the Californian *Heyderia decurrens* (Torr.) K. Koch (*Libocedrus decurrens* Torr.) carvacrol, hydrothymoquinone, thymoquinone, the two hydrothymoquinone monomethyl ethers, libocedrol (which is a dehydrogenation product of one of them) and (Dr. Anderson, private communication) β-thujaplicin. *Heyderia*, therefore shows certain chemical affinities to the northern *Tetraclinis* from which Grimal [3] isolated the three first mentioned compounds but which, generally, has been regarded to be related to a southern tribe of the Cupressaceae. The East Asian *Heyderia formosana* (Florin) Li (= *Libocedrus formosana* Florin) which was studied some time ago by Ishikawa [4], apparently, differs considerably from *H. decurrens* containing mainly a series of highly interesting terpenoid acids among which shonanic acid is the best known. The presence of unidentified "phenols" was also reported.

We have now examined the heartwood of *Austrocedrus chilensis* (D. Don) Florin et Boutelje *(Libocedrus chilensis* D. Don) which is endemic to Chile and constitutes the sole recent species of the genus *Austrocedrus*. Taxifolin and β-thujaplicin were isolated. This is the first instance of a tropoloniferous member of the family Cupressaceae occurring in the southern hemisphere. The yield of β-thujaplicin was unexpectedly high (over two per cent) and the wood, therefore, constitutes a rich source of this tropolone. As one would expect considering the fact that the "phenol coefficient" of β-thujaplicin [5] is over 100, the wood is extremely durable and resistent to the attack of wood destroying fungi (Docent E. Rennerfelt, private communication).

Experimental. 1. *Extraction with acetone.* The powdered heartwood (1 240 g) was extracted continuously for three days with acetone. The soluble material (151 g) was shaken three times with ether (500 ml portions) yielding an ether soluble fraction (79 g), "A". The residue was boiled twice with water (300 ml portions) for 15 minutes and the combined extracts treated with animal charcoal, cooled and shaken with ether. The ether solution was dried over sodium sulphate and the ether removed. The residue was boiled with chloroform (10 ml) and the solution cooled when taxifolin (1 g) crystallised. Solution A was evaporated to dryness and the residue boiled with ligroin (b. p. 90—120°) (3 × 500 ml) when 38 g material passed into solution ("B") leaving an insoluble residue (41 g) which was worked up like A employing, however, 30 ml of chloroform. 3 g Taxifolin was isolated followed by a second crop (0.3 g) on concentration of the mother liquor. Total yield of crude taxifolin 4.3 g (0.35 %). It was purified by recrystallisation from water, m. p. 238—239°, (not depressed on admixture with an authentic specimen), $[\alpha]_D^{24}$ 47° (acetone-water 1:1, *c* 1.03). The purity of the substance as well as the absence of aromadendrin in the mother liquors was demonstrated by paperchromatography em-

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ploying 1) neutral paper, 2) boric acid impregnated paper, and as developers 1) hypophase of chloroform, ethanol, water (8:2:1) and 2) epiphase of benzene, ethanol and boric acid saturated water (6:2:1). Only a single spot corresponding to that of taxifolin was observed on spraying with bisdiazotised benzidine.

The ligroin solution B was evaporated and the residue dissolved in ether (700 ml) and shaken successively with a saturated aqueous solution of sodium bicarbonate (3 × 200 ml), a 10 % sodium carbonate solution (4 × 300 ml) and a 5 % potassium hydroxide solution (3 × 200 ml). Evaporation of the residual ether solution yielded a neutral fraction (6 g) which was subjected to distillation with steam. Only about one gram of a terpene-like volatile oil was obtained and a semisolid non-volatile fraction which were not further investigated.

Acidification and ether extraction of the aqueous solutions yielded a bicarbonate soluble fraction (1.1 g), a "carbonate fraction" (26 g) and an "alkalifraction" (1.8 g) of which only the carbonate fraction was investigated. It crystallised readily and yielded after recrystallisation from light petroleum (b. p. 40—60°) pure β-thujaplicin (25.8 g, 2.2 %) m. p. 52.5—53° (mixed m. p.). Cu-complex: from chloroform crystals m. p. 93—94° (containing solvent of crystallisation) resolidifying and again melting at 165.5—165.7° Paperchromatographic tests employing sodium borate impregnated paper and butanol, ethanol, water (5:1:4) or benzene, ligroin (b. p. 80—120°) as developers indicated the absence of carvacrol in the mother liquor. There was an extremely weak spot corresponding to α-thujaplicin.

2. *Extraction with ligroin.* The ground wood (100 g) was extracted continuously with hot ligroin for 15 hours. The extract (2.3 g) was an oil which easily crystallised. By dissolution in dilute potassium hydroxide solution and precipitation with hydrochloric acid a yield of 1.6 g of practically pure β-thujaplicin was obtained.

3. *Extraction with alkali.* The ground wood (100 g) was mixed with a 2 % solution of potassium hydroxide (500 ml). After fifteen minutes the mixture was filtered and this operation repeated twice. The combined extracts were acidified with hydrochloric acid and shaken with ether. In this manner a fraction insoluble in ether (4 g) and a fraction soluble in ether were obtained. The latter was distilled *in vacuo* (10 mm) yielding a volatile product (2.5 g) which crystallised directly and from which pure β-thujaplicin (2.1 g) was isolated.

We are greatly indebted to the *Scientific Department, Israeli Ministry of Defence,* for financial support to one of us (Z. P.) and to Jägmästare I. Lagerborg, Talca, Chile, for providing the wood.

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The Diastereomers of α-Methyl-α′-ethyl-glutaric Acid

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In connection with an investigation into configurative relations in progress at this institute Schotte [1] has studied the infrared absorption spectra of the two isomers of α-methyl-α′-ethyl-glutaric acid. As early as 1946 these isomers were prepared by the author and this communication describes the method used.

The separation of the two isomers was performed by making use of the different solubility of their acid calcium salts and after several recrystallisations from water they melted at 83.5—84.5° and 105.5°, respectively. According to Schotte [1] the configuration of the low melting acid is probably mesoid and that of the high melting racemoid. On mixing the two isomers in about equal quantities, the mixed melting point was 62.5—64°. Owing to shortage in material only a few mixed melting points could be obtained but these suggest an eutectic at about 62° with approximately 40 % high melting acid.

Another method of separation has been used by previous authors [2-4], *viz.* by converting the mixture of diastereomeric α-methyl-α′-ethyl-glutaric acids to the imides which are then separated and hydrolysed. The high melting acid thus prepared was reported [2] to melt at 107°, the low melting product must, however, still have been a

ploying 1) neutral paper, 2) boric acid impregnated paper, and as developers 1) hypophase of chloroform, ethanol, water (8:2:1) and 2) epiphase of benzene, ethanol and boric acid saturated water (6:2:1). Only a single spot corresponding to that of taxifolin was observed on spraying with bisdiazotised benzidine.

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mixture of the two acids according to the melting points given [2-4]: 63°, 70—71° and 72—73°.

Preliminary experiments indicated that the resolution of the two α-methyl-α'-ethyl-glutaric acids into optical antipodes could be made by means of their strychnine salts.

Experimental. Methyl ethyl-glutaric acid was prepared in two ways:

1. By adding methyl methacrylate to an alcoholic solution of diethyl ethylmalonate in the presence of sodium ethoxide, methyl ethyl 1-ethyl-3-methyl propan-1,1,3-tricarboxylate boiling at 160—162°/7 mm was formed (45 %).

2. By condensing ethyl α-bromoisobutyrate with diethyl ethylmalonate according to Rydon [2], when diethyl 1-ethyl-3-methylpropan-1,1,3-tricarboxylate is formed (68 %).

The methyl ethyl glutaric acid was obtained by refluxing these esters with an excess of constant boiling hydrochloric acid for about 25 hours, evaporating to dryness and heating the residue at 170° during three hours. The resulting mixture of isomers melted at 67—74° and was recrystallised from water.

Several experiments indicated, that the best method for separating the two isomers was to dissolve the mixture in five times its own weight of water and at 40—50° add calcium carbonate in an amount corresponding to 75 % of what is required for the acid calcium salt. From the precipitate the high melting acid was obtained after acidification and extraction with ether and melted, after several recrystallisations from water, at 105.5°. [Found: equiv. wt. (by titration) 87.1. $C_8H_{14}O_4$ requires equiv. wt. 87.1]. The low melting acid was prepared in the same way from the filtrate and gave, after recrystallisations from water, a melting point of 83.5—84.5°. [Found: equiv. wt. (by titration) 87.4. $C_8H_{16}O_4$ requires equiv. wt. 87.1]. 28.7 g mixture gave 11.35 g high melting acid and 15.4 g low melting acid.

Before taking the mixed melting points the acids were dissolved in dry acetone and after evaporation dried in vacuum over P_2O_5.

% High melting acid	m. p.
27.4	62—68°
52.1	64—73°
76.6	81—91°

Professor Arne Fredga suggested this investigation and I take this opportunity of expressing my sincere gratitude for his valuable help and advice.

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